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Phosphatase Epsilon in Mammary Tumor Cell Lines

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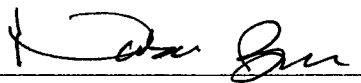
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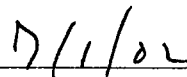
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Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.**12b. DISTRIBUTION CODE****13. ABSTRACT (Maximum 200 Words)**

Previous studies have established a connection between overexpression of the receptor-type tyrosine phosphatase Epsilon (PTPe) and mammary tumors induced in mice by the Neu oncogene. This project aimed to understand the role of PTPe in mammary tumorigenesis by identification of physiological substrates of PTPe and by understanding the effect of PTPe on their function. In the course of these studies we constructed several substrate-trapping mutants of PTPe, which can bind to and help isolate PTPe substrates. Trapping mutants showed, and additional biochemical and cellular studies confirmed, that the delayed-rectifier, voltage-gated potassium channels Kv1.5 and Kv2.1, as well as the Src tyrosine kinase, are physiological substrates of PTPe. Examination of PTPe-deficient mice revealed that lack of PTPe correlated with hyperphosphorylation and activation of Kv1.5 and K2.1, as well as with severe transient hypomyelination of sciatic nerves of young post-natal mice. Mammary tumors induced by Neu in PTPe-deficient mice exhibited altered Src phosphorylation and activity and grew poorly in culture and in vivo. These and other experiments outlined here indicate that PTPe is a physiological inactivator of Kv channels and a physiological activator of Src in vivo.

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4. Introduction:

The study summarized here focused on providing molecular-level understanding the role of protein tyrosine phosphatase epsilon (PTPe) in the genesis of breast cancer by identifying physiological substrates of the enzyme. We have previously demonstrated a link between PTPe and genesis of mammary tumors in mice *in vivo*; the project supported by the USAMRMC was aimed at taking these findings one step further by defining what is one of the most basic items of information needed to understand the roles of PTPe - the identities of its substrates. A major facilitator in achievement of this goal was use of substrate-trapping methodology, i.e., use of PTPe mutants which lack catalytic activity but which are able to bind their phosphorylated substrates and remain bound to them during purification. In the course of this project we constructed and characterized the necessary mutant PTPe molecules and performed studies in which we attempted to verify that specific candidate substrates of PTPe were indeed substrates of the enzyme, as well as studies in which we used substrate-trapping mutants for isolation of substrates without prior attempts to guess their identities. Our studies have resulted in identification of three substrates for PTPe: the delayed rectifier, voltage-gated potassium channels Kv1.5 and Kv2.1, and the Src tyrosine kinase. Functional significance of PTPe acting on these molecules was revealed in studies utilizing gene-targeted mice lacking PTPe. These studies revealed that lack of PTPe causes severe transient hypomyelination in peripheral nerves, which is correlated with increased phosphorylation and activation of the Kv1.5 and Kv2.1 channels. Examination of mammary tumors induced in PTPe-deficient mice by the Neu oncogene has revealed that lack of PTPe causes altered phosphorylation and a drop in activity of Src in these tumors. Cells derived from PTPe-deficient tumors appear morphologically less transformed and grow relatively poorly, both in culture and after injection into nude mice. Expressing Src in these cells rescues the morphology and proliferation rate phenotypes. These findings indicate that PTPe is a physiological activator of Src *in vivo*, and that inhibition of PTPe can result in partial inhibition of Src. As Src is an important co-factor in Neu-induced mammary tumorigenesis, this finding suggests that inhibitors of PTPe would indirectly inhibit Src in a way which might be clinically useful in fighting breast cancer.

5. Body of report:

A. Outline of studies planned, based on the approved Statement of Work (SOW):

The project consisted of the following stages, abbreviated from the approved SOW:

1. Technical setup:

Construction of a series of bacterial expression plasmids for expression of GST-PTPe mutant and wild-type fusion proteins, expression of above plasmids in bacteria, isolation of GST-PTPe fusion proteins. Construction of analogous expression vectors for eukaryotic cell systems.

2. Use of substrate-trapping mutants:

Visualization of hyperphosphorylated proteins which bind to GST-PTPe fusion proteins in extracts of mouse mammary tumor cells and mouse tissues. Trapping substrates by expressing trapping mutants of PTPe in mammary tumor cell lines. Isolation of proteins which bind trapping mutants, microsequencing them, search in databases for their identity. Proof that these proteins are indeed substrates by specifically inhibiting their association with PTPe through use of sodium pervanadate.

3. Study of substrates:

Resource building (antibodies, expression constructs, etc. for substrates). Analysis of function of substrate in breast cancer cell lines and PTPe-deficient mice. Analyses include protein expression levels, phosphorylation, identification of stimuli which govern phosphorylation and function of substrates, identification of specific residues targeted by PTPe in these substrates, physiological function in mice.

B. Brief overview of substrate-trapping technology:

Substrate-trapping mutants of protein tyrosine phosphatases (PTPs) are PTP molecules in which specific mutations have been introduced such that the resulting molecules are either almost or entirely devoid of catalytic activity, but retain the ability to bind their phosphorylated substrates and to remain bound to them (Sun et al., 1993; Flint et al., 1997). Binding is typically strong enough to withstand the rigors of immune-precipitation or related procedures, making substrate-trapping mutants "affinity reagents" of sorts for identifying physiological substrates of the phosphatase at hand. Two generic substrate-trapping mutations in PTPases have been described - mutation of the cysteine residue located at the core of the catalytic domain to a serine residue (C-to-S mutation), or mutation of a nearby aspartic acid residue to an alanine (D-to-A) (Flint et al., 1997; Sun et al., 1993). Both residues are strictly conserved in all known PTPases, making this approach systematically applicable to the entire PTPase family. Both types of reagents

are useful to similar extents in trapping studies, although the technical details of their production and utilization differ somewhat.

C. Construction of GST-PTPe fusion molecules:

Bacterial expression vectors in which the entire cytoplasmic domain of tm-PTPe, containing both catalytic domains (D1&D2), or just the membrane-proximal (D1) catalytic domain of the enzyme were constructed in the pGEX2TK plasmid (Pharmacia). The required fragments of PTPe were amplified from tm-PTPe cDNA (clone 58, Elson and Leder, 1995a) by PCR, using the e5common oligomer (GGGGATCCAGGTTCCGGAAGCAGAGGA), together with either the AEF3.1 oligomer (TGAAGTCTTCCTCCAGCCCG, for the D1 construct) or the Y695/NHE oligomer (GGGCTAGCTCATTTGAAATTAGCATA, for the D1&D2 construct) (See Figure 1). D-to-A mutants, in which the aspartic acid of the D1 domain was mutated to an alanine, were created by site-directed mutagenesis (Kunkel, 1985). The relevant DNA fragments were cloned into the BamHI/SmaI sites of pGEX2TK; the resulting plasmids were expressed in the BL-21 strain of *E. coli* bacteria.

GST-PTPe fusion proteins were purified from crude bacterial lysates using glutathione-agarose beads (Pharmacia). Expression of GST-PTPe fusion proteins was verified by protein blotting of extracts of bacteria with anti-PTPe antibodies, and by Coomassie blue staining of SDS-polyacrylamide gels through which the lysates or purified proteins had been electrophoresed (Figure 2). In general, protein blot analysis revealed that the GST fusion proteins indeed reacted with anti-PTPe antibodies, indicating that the bacterial expression vectors drove expression of the correct protein. However, Coomassie blue analysis of the purity and of the size of the GST-fusion proteins indicated that full-length GST-PTPe proteins were not being produced in an adequate manner. The protein preparations contained, in addition to full-length GST-PTPe protein, shorter proteins which were likely products of specific cleavage and non-specific degradation (Figure 2). Problems of this nature are often associated with production of GST fusion proteins, especially long ones, as was the case here.

We attempted to overcome these difficulties by optimizing bacterial growth conditions. Several strains of *E. coli* bacteria were used to try and find one optimal for production of GST-PTPe proteins. None of the strains (BL21, BL26, DH5, XL1-Blue) produced a significant change in yield of full-length GST-PTPe protein. We also attempted to limit protein degradation by slowing down the rate of bacterial growth, by growing bacteria in relatively poor media, at temperatures lower than the standard 37 degrees, or by reducing shaking (and aeration) of bacterial cultures. None of these

attempts significantly improved the situation. This resulted in our decision to focus mainly on trapping studies by expressing PTPe mutants in eukaryotic systems, provisions for which had been made in the original research proposal. We intend, however, to eventually use the GST fusion constructs for this purpose as well.

D. Construction of D-to-A mutants of PTPe in eukaryotic expression vectors.

The desired D-to-A mutations were inserted into the cDNAs of the two major forms of PTPe - transmembranal PTPe (tm-PTPe; Elson and Leder, 1995a) and cytoplasmic PTPe (cyt-PTPe; Elson and Leder, 1995b). Mutations were inserted using the Kunkel technique for site-directed mutagenesis (Kunkel, 1985); presence of the desired mutations and absence of other sequence changes were verified by sequencing. Both mutant cDNAs (D302A tm-PTPe and D245A cyt-PTPe) were cloned into the pCDNA3 expression vector, and their expression in 293 and COS cells was verified by transient expression and protein blotting with anti-PTPe serum (Elson and Leder, 1995a) (Figure 3).

D-to-A mutants of tyrosine phosphatases are known to be virtually inactive (Flint et al., 1997). In order to determine whether this was the case with our series of D-to-A mutants of PTPe, we compared total tyrosine-phosphatase activity in cells into which we had transfected wild-type PTPe or D-to-A mutants of PTPe (Figure 4). Experiments consisted of transiently transfecting the relevant plasmids into 293 cells, followed by lysing the cells in an NP-40-based buffer. Equal amounts of protein were incubated together with paranitrophenyl-phosphate (PNPP), a substrate for phosphatase activity. Dephosphorylation of PNPP yields a product which absorbs light at 405nm. Phosphatase activity was then followed as the change in OD405 as a function of time elapsed (up to 120 minutes).

PNPP can be the substrate also of non tyrosine-specific phosphatases. To correct for this, each experiment was repeated also in the presence of 1mM sodium pervanadate, a powerful, irreversible, and specific inhibitor of tyrosine phosphatases (Huyer et al., 1997; Flint et al., 1997). The rate of PNPP dephosphorylation in the presence of pervanadate was taken to represent activity of non tyrosine-specific phosphatases and was subtracted from the total activity measured without pervanadate. PTPe activity was taken to be the difference between PTP activity in transfected cells minus PTP activity in mock-transfected cells. PTPe expression levels in transfected cells were verified by protein blotting.

As seen in Figure 4, all D-to-A mutants of tm- or of cyt-PTPe were entirely inactive, in agreement with our expectations. Presence of a FLAG tag at the extreme

carboxy-terminus of PTPe molecules did not affect catalytic activity. Similar results were later obtained in assaying the ability of wild-type or of mutant PTPe to inhibit activity of voltage-gated potassium channel activity in *Xenopus* oocytes (see below). This series of mutants was and is currently being used to verify that candidate substrates of PTPe are in fact substrates of the enzyme, as well as in experiments aimed at isolating substrates of PTPe from mammary tumor cell lines without prior attempts to guess their identity.

E. Use of D-to-A mutants of PTPe:

1. Identification of voltage-gated potassium channels as physiological substrates of PTPe. (This study has been published as Peretz et al., 2000. Although the study is described here in detail, reference is made to figures included in the paper itself, which is included as an appendix).

In parallel to the research effort described above, we had been characterizing the phenotype of PTPe-deficient, knockout mice we had produced and had come across a defect in myelination of axons in their peripheral nerve system. We had evidence to suggest that this defect was due to aberrant phosphorylation of voltage-gated potassium (Kv) channels in Schwann cells of these mice, and wished to use the substrate-trapping system described above to determine whether Kv channels were in fact substrates of PTPe. Although the studies which led to this point fell outside the scope of the current project, we felt that use of substrate-trapping mutants was nonetheless highly relevant to the goals of the study reported here. The Kv channel study provided us with an excellent opportunity to verify the functionality of the reagents we had constructed and of our operating procedures, using a defined candidate substrate of PTPe, which preliminary evidence indicated was very likely to turn out to be a physiological substrate of PTPe. Furthermore, Kv channels are quite widespread and are expressed in many organs and tissue types which are not conceived as being electrically excitable (Lewis and Cahalan, 1995), at the time raising the possibility that Kv channels might be relevant substrates of PTPe also in the context of breast cancer.

Previous findings relating to Kv channels in PTPe-deficient mice: In the process of characterizing PTPe-deficient mice we performed an electron microscopy-based study of the thickness of myelin sheaths surrounding axons in the sciatic nerves of these mice. The original rationale for performing this study was based on knowledge that myelination of peripheral nerves is dependent upon the well-being and functioning of Schwann cells, that Schwann cell proliferation and function is strongly influenced by the functioning of Kv

channels expressed in them, and that Kv channels, in turn, can be regulated by Src- and Fyn-mediated tyrosine phosphorylation (Peretz et al., 1999). Several studies documenting functional connections between Src and Fyn, on the one hand, and PTPa, a PTPase extremely closely related to PTPe, on the other hand, have been published (Zheng et al., 1992; Su et al., 1999; Ponniah et al, 1999). This suggested to us that perhaps PTPe could be involved in regulation of Kv channel function in Schwann cells, and that this may be manifested in abnormal myelination of axons.

We first established that primary Schwann cells do in fact express PTPe, cyt-PTPe in particular (Figure 1, Peretz et al, 2000). We then examined the extent of myelination in PTPe-deficient sciatic nerve axons by electron microscopy and found it to be severely reduced compared to wild-type samples (Figure 2, Peretz et al, 2000). Further studies revealed that alpha subunits of Kv channels in primary Schwann cells derived from PTPe-deficient mice were hyperphosphorylated on tyrosine residues when compared to age-matched controls (Figure 3, Peretz et al, 2000). Furthermore, patch-clamp measurements performed on primary Schwann cells revealed that Kv channel activity was markedly elevated in PTPe-deficient cells (Figure 3, Peretz et al, 2000). Together, these results indicated that lack of cyt-PTPe caused hyperphosphorylation and up-regulation of Kv channels, and most likely accounted for reduced myelination observed *in vivo*.

From these findings we deduced that a normal role of cyt-PTPe in Schwann cells is to lower tyrosine phosphorylation levels and activities of Kv channel alpha-subunits. This conclusion was strengthened by the ability of cyt-PTPe to reduce Src-mediated phosphorylation of Kv2.1 *in vivo* (Figure 6, Peretz et al., 2000). PTPe could presumably act either directly, by dephosphorylating Kv channel proteins, or indirectly, by dephosphorylating and inactivating kinases (chiefly Src and Fyn), which phosphorylate and activate Kv channels (Peretz et al., 1999). Further studies revealed that activities and phosphorylation levels of Src and of Fyn were similar in Schwann cells of PTPe-deficient and of wild-type mice, strongly suggesting that Src and Fyn were not major mediators of the effect of cyt-PTPe on Kv channels. This raised the possibility that PTPe directly dephosphorylates Kv channel alpha subunits.

Use of PTPe substrate-trapping mutants: In order to determine whether Kv channel alpha subunits were in fact substrates of PTPe, we made use of the D-to-A mutants of PTPe described above. The purpose of this experiment was to determine whether the major Kv channel alpha-subunit found in Schwann cells, Kv2.1, would specifically bind the D-to-A mutant of PTPe at the latter's active site. For this purpose,

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we transiently expressed Kv2.1 in 293 cells together with wild-type or the D-to-A mutant of PTPe. PTPe was immune-precipitated using anti-FLAG antibodies, and presence of associated Kv2.1 was examined by protein blotting with anti-Kv2.1 antibodies. As seen in Figure 7 (Peretz et al, 2000), low levels of Kv2.1 reproducibly precipitated with wild-type PTPe. However, significantly more Kv2.1 co-precipitated with the D-to-A mutant of PTPe, indicating that the D-to-A mutation had significantly increased the ability of PTPe to bind Kv2.1. Furthermore, this increased binding was eliminated by the presence of sodium pervanadate in the precipitation reaction. This last finding was critical, as pervanadate oxidizes the cysteine residue located at the catalytic center of PTPases; in the case of D-to-A mutants, this is known to prevent association between the active site of the mutant molecules and their putative substrates (Flint et al., 1997). Together, these experiments clearly demonstrated that the interaction between Kv2.1 and PTPe was mediated by the active site of the phosphatase, a finding which was clearly consistent with Kv2.1 being a substrate of PTPe.

In line with the above results, expression of wild-type PTPe together with Kv2.1 in *Xenopus* oocytes severely reduced voltage-gated potassium currents caused by Kv2.1, while the D-to-A mutant of PTPe had little effect on Kv2.1 activity (Figure 6, Peretz et al, 2000). This indicates that the D-to-A mutant PTPe molecule is in fact catalytically inactive also as judged in a functional assay which is more specific and physiological than the PNPP dephosphorylation assay described above. Residual reduction in Kv2.1 activity caused by D-to-A PTPe can be readily explained by mutant PTPe binding to some Kv2.1 molecules and thus preventing them from functioning. This effect is expected to be much weaker than enzymatic dephosphorylation of Kv2.1 molecules by active PTPe, as the latter effect is catalytic, while binding is a stoichiometric process in which each PTPe molecule can affect only a single Kv2.1 molecule.

Identification of the site of dephosphorylation by PTPe in Kv2.1

Data presented above indicated that in dephosphorylating Kv2.1, cyt-PTPe was in effect countering the activity of Src towards this channel molecule. Kv2.1 has 19 tyrosine residues, of which 14 are in parts of the molecule which are cytoplasmic, i.e., to which Src would be expected to have access. Furthermore, the consensus sequence for phosphorylation by Src is known to be XEITYXE, corresponding nicely with the sequence present at positions 121-126 of rat Kv2.1 (DEIYLE, Sobko et al., 1998). The data suggested that the tyrosine targeted by PTPe would probably be part of a Src consensus

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sequence, hence we decided to examine the possibility that Y124, the only tyrosine present in the above sequence, was the substrate of both Src and PTPe. In order to achieve this goal, we mutated Y124 in Kv2.1 to an unphosphorylatable phenylalanine residue.

As seen in Figure 5, Y124F Kv2.1 was significantly less phosphorylated by Src in intact cells. Phosphorylation was reduced by approximately 60%, indicating that this residue is indeed the major site of phosphorylation by Src in Kv2.1. Co-expressing wild-type PTPe together with Src in cells expressing either wild-type or Y124F Kv2.1 reduced Kv2.1 phosphorylation to similar background levels, indicating that the residual 40% of Src-mediated phosphorylation in Kv2.1 was also a target of PTPe (not shown)

Further studies indicated that the Y124F mutant of Kv2.1 was significantly less able than wild-type Kv2.1 to bind the D-to-A substrate-trapping mutant of cyt-PTPe (Figure 6). Binding was reduced by ~65%, i.e. by approximately the same extent that phosphorylation by Src of Y124F Kv2.1 was reduced. This last result is crucial, as it directly shows that Y124 is the major site to which the active site of PTPe binds, consistent with PTPe-mediated dephosphorylation occurring at this site. The implications of PTPe-mediated dephosphorylation of Kv2.1 on channel activity are currently being examined using electrophysiological approaches of the type described in Peretz et al., 2000.

2. PTPe as a physiological activator of Src; Molecular mechanism of PTPe promotion of mammary tumors *in vivo*.

In addition to proving the utility of substrate-trapping mutants of PTPe and allowing us an opportunity to calibrate our working procedures, the above study of Kv channels also demonstrated the potential offered by successful "educated guesses" of the identities of PTPe substrates. All of these considerations were brought to bear in the subsequent identification of Src as a substrate of PTPe in mammary tumor cells.

Establishing PTPe-deficient mammary tumor cell lines

In a study unrelated to the present project, gene-targeted mice lacking PTPe had been mated with MMTV-Neu transgenic mice, in which the Neu transgene was expressed in the mammary epithelium due to the activity of the MMTV (Mouse Mammary Tumor Virus) promoter/enhancer, giving rise to mammary tumors (Muller et al, 1988). We had previously established that the receptor-type form of PTPe (tm-PTPe) plays an

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accessory role in this process, as mammary tumors induced specifically by either Neu or Ras exhibit significant upregulation of PTPe (Elson and Leder, 1995a). Furthermore, transgenic mice expressing PTPe in their mammary epithelium develop mammary hyperplasia and associated tumors (Elson, 1999). The mating described above was performed in order to examine whether the role PTPe plays in this process is necessary for the ability of Neu to transform mammary epithelial cells.

Neu-induced tumor incidence in mice was similar irrespective of whether the mice did or did not contain a functional PTPe gene. We present the tumor-free survival curve of these mice as Figure 7 for clarity, even though this result is not part of the study being reported here. However, we reasoned that substrates of PTPe could be phosphorylated differently in Neu-induced mammary tumors, dependent upon whether PTPe was or was not expressed in these tumors. We further reasoned that cell lines derived from these tumors would be valuable assets in identifying substrates of PTPe by either the "educated guess" or the substrate-trapping approach.

In order to examine this possibility, we established several of the aforementioned mammary tumors in culture as cell lines. Lines were established by isolating tumor tissue from mice, finely mincing it in tissue culture dishes in culture medium (DMEM/10% bovine serum/ 2mM Glutamine/ 100 units/ml penicillin/ 100 mg/ml streptomycin), and passaging the cells until a healthy population of uniform epithelial morphology arose. The cell lines in question all arose on the same genetic background (50% FVB/N, 50%C57Bl6/129) and all were transformed due to the activity of the Neu protein, as no tumors arose in mice which did not carry the MMTV-Neu transgene (not shown). However, with respect to PTPe, three groups of tumors could be distinguished - tumors which contained both alleles of PTPe (wild-type, WT), tumors which arose in mice heterozygous for the PTPe null alleles, and tumors which arose in mice lacking both alleles of PTPe mice. In the studies outlined below, tumor cells expressing one or two functional alleles of PTPe were indistinguishable from one another and are referred to as Neu tumor cells. Cells from PTPe-deficient tumors are referred to as EKO/Neu cells.

Properties of PTPe-deficient mammary tumor cells

All cell lines expressed the Neu transgene; high levels of endogenous tm-PTPe protein were expressed only in cells from mice carrying at least one functional allele of PTPe (Figure 8A). Note that all EKO/Neu and Neu tumor cells expressed significant and similar amounts of PTPa, a PTP closely related to PTPe. As seen in Figure 8B, cells from

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tumors which arose in WT mice (i.e., mice which expressed the Neu transgene and the normal WT complement of PTPe) were epithelial in morphology, possessed prominent nuclei, and exhibited compact, cobblestone-like morphology. In contrast, cells from similar tumors which arose in mice lacking both alleles of PTPe were much more flat and spread out, although they were also of clear epithelial morphology. Importantly, EKO/Neu cells proliferated significantly slower than Neu cells (Table 1). No differences in cell survival or plating efficiencies were observed between EKO/Neu and Neu tumor cells indicating that slower proliferation rate of EKO/Neu cell cultures was due to slower proliferation of these cells. EKO/Neu cells also tended to form smaller colonies than Neu cells when grown in soft agar, but variability among lines prevented this phenomenon from reaching statistical significance (Table 1 and results not shown).

Differences in growth rates noted above persisted *in vivo*, following injection of EKO/Neu or Neu tumor cells into the mammary fat pad or subcutaneously into the flank of nude mice. 15 days following injection mice were sacrificed and the tumors excised and weighed. EKO/Neu and Neu tumor cells generally formed tumors *in vivo*, with tumors formed in the mammary gland significantly larger than those of the flank. Interestingly, tumors which arose from EKO/Neu cells were significantly smaller than Neu-based tumors in both the mammary fat pad (-78%) and flank (-55%) (Table 1); similar results were obtained in separate experiments following a 21 day incubation period (not shown). Tumors which arose in nude mice lacked any visible signs of necrosis and had clearly succeeded in recruiting blood vasculature, arguing that reduced growth of EKO/Neu tumors *in vivo* was not due to differences in cell survival or angiogenesis. Of the cell lines examined, only the Neu cell line 1904 did not form tumors *in vivo*, a phenomenon observed in six separate injections into nude mice and after follow-up periods significantly longer than those noted here. The inability of line 1904 to form tumors *in vivo* is therefore unique to this line and is not characteristic of the Neu genotype. We conclude that tm-PTPe is required for proper development of Neu-induced tumor cells both *in vitro* and *in vivo*, and that in its absence cells develop poorly.

Src as a substrate of PTPe

The above studies clearly indicated that lack of PTPe had rendered Neu-induced mammary tumor cells "less well off", most likely due to altered phosphorylation and dysregulation of a substrate of PTPe. The Src tyrosine kinase is a major candidate for being such a substrate, since Src is a known collaborator of Neu in transformation of mouse mammary epithelial cells (Dankort and Muller, 2000; Musuthwany and Muller,

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1995). Furthermore, Src can be dephosphorylated and activated by the related PTPa *in vitro* and *in vivo* (Su et al., 1999; Ponniah et al., 1999). We therefore asked whether lack of tm-PTPe could affect Src activity in mammary tumor cells, thereby causing the EKO/Neu phenotype. Preliminary evidence in support of this hypothesis was obtained from protein blotting experiments using phospho-specific antibodies, which revealed that Src phosphorylation at its C-terminal inhibitory site Y527 (numbering as in chicken Src), was increased by 51% in EKO/Neu cells, while autophosphorylation at Y416 was reduced by 63% (Figure 9). Both changes are typically associated with reduced Src kinase activity (Abram and Courtneidge, 2000); indeed, direct measurements revealed a two-fold reduction in Src activity in lysates of EKO/Neu cells (Figure 10).

We next examined whether Src could bind D-to-A substrate-trapping mutants of PTPe. The molecule used here was the D302A mutant of the receptor-type form of PTPe (tm-PTPe), the isoform of PTPe found in Neu-induced mammary tumor cells (Elson and Leder, 1995a). Trapping studies were performed in SYF cells, which are SV40-transformed embryo fibroblasts genetically deficient for the Src, Yes, and Fyn tyrosine kinases (Klinghoffer et al., 1999). SYF cells were used so that no interference from endogenous Src from non-transfected cells would be encountered. SYF cells do not express PTPe (not shown).

Following co-expression of Src with wild-type tm-PTPe in SYF cells, Src was immune-precipitated and blotted to reveal associated PTPe. Small amounts of WT tm-PTPe specifically associated with Src, and were not detected in identical experiments from which the primary precipitating anti-Src antibody was omitted (Figure 11). Replacing WT tm-PTPe with the trapping mutant D302A tm-PTPe resulted in significantly more tm-PTPe being co-precipitated with Src; again, binding was specific and was not detected in the absence of the precipitating antibody (Figure 11). No binding of PTPe to the anti-Src antibody was observed in experiments where PTPe was subjected to precipitation by the anti-Src antibody in the absence of co-transfected Src (not shown). These results indicate that Src interacts with the active site of tm-PTPe, consistent with Src being a substrate of tm-PTPe. The analogy with PTPa, which also binds Src, suggests that the interaction between Src and PTPe is not limited to the active site of PTPe (Zheng et al., 2000). This issue is currently being examined.

In order to determine whether PTPe could actively affect the phosphorylation and activity of Src (i.e., whether lack of PTPe in EKO/Neu tumor cells merely correlated with or could be the cause of altered Src phosphorylation and activity), we examined the effect

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of overexpressing PTPe on Src in transfected cells. These studies were conducted in SYF cells as well. As expected, co-expression of Src and of tm-PTPe resulted in changes in Src which were opposite from those observed in the PTPe-deficient EKO/Neu cells: Y527 phosphorylation was decreased by 27%, Y416 phosphorylation was increased by 52%, and Src activity was increased by 78% (Figures 12A, 12B, and 12C). Similar results were obtained in cells transfected with Src and PTPa (not shown). These results are consistent with tm-PTPe preferentially dephosphorylating Src at Y527, thereby activating the kinase and resulting in increased autophosphorylation at Y416. Interestingly, the non receptor-type form of PTPe, cyt-PTPe, strongly reduced pY527 levels in transfected SYF cells by 66% (Figure 12A and 12B). Src activity was increased by 117% by cyt-PTPe (Figure 12C), although no changes in pY416 levels were detected (Figures 12A and 12B). We interpret this as being due to the stronger cyt-PTPe activity in these experiments partially dephosphorylating Y416 of Src, thereby countering autophosphorylation at this site. Note that similar levels of tm-PTPe and full-length cyt-PTPe were expressed in these cells; p67 PTPe and p65 PTPe, which are significantly co-expressed with cyt-PTPe, are exclusively cytosolic proteins and do not reduce phosphorylation of Src (Gil-Henn et al., 2000; Gil-Henn et al., 2001; and unpublished results). A detailed comparison of the effects of tm-PTPe and cyt-PTPe on Src is currently underway.

Increasing Src activity can rescue the phenotype of PTPe-deficient mammary tumor cells.

We next examined whether added expression of Src in EKO/Neu cells could rescue some aspects of the phenotype of these cells, thereby supporting a PTPe-Src-phenotype connection. An assumption inherent in this line of study was that some aspects of the EKO/Neu phenotype are in fact reversible. This is a non-trivial assumption as these cells were derived from tumors, which had undergone extensive selection *in vivo* and may have progressed beyond the point of phenotype reversibility. Nonetheless, we examined EKO/Neu cells which had been infected with retroviral vectors expressing constitutively active (Y527F) Src or WT Src. Similar cells infected with empty vector served as controls in these experiments. WT Src and Y527F Src were detected in infected cells by protein blotting (Figure 13A); expression of exogenous Y527F Src was lower than that of exogenous WT Src possibly due to harmful long-term effects of massive overexpression of this highly-active Src mutant.

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Examination of the morphology of the infected cells revealed that cells expressing either WT or constitutively active Src acquired most morphological characteristics found in Neu cells, such as smaller size and denser growth, and a less-flattened morphology. These changes were not detected in cells infected with empty viral vector, indicating that they were indeed caused by exogenous Src (Figure 13B). Expression of constitutively active Src in EKO/Neu cells also significantly increased the rate of cell proliferation as compared with cells expressing WT Src or infected with empty vector (Figure 13C). The differences between the effect of WT Src on cell shape vs. cell proliferation rates are likely due to each process being regulated in a distinct manner by Src.

Attempts to correct the EKO/Neu cellular phenotype by expressing tm-PTPe yielded inconclusive results. Following infection of line 7381 cells with a retroviral construct for tm-PTPe, Src phosphorylation at Y527 and Y416 were reduced by 19.8% and 45%, respectively, in partial agreement with results presented above (not shown). Morphology and growth rates of these cells were not affected in a consistent manner. We attribute these results in part to the possibility that the phenotype of these cells, which had undergone intense selection *in vivo* during tumorigenesis, may have progressed beyond the point of reversibility by tm-PTPe expression. It might also be possible that, as we move further away from Src, the efficiency with which genetic changes, such as expression of PTPe, affect the cellular phenotype is reduced.

Conclusions from the Src/PTPe/mammary tumor study:

1. tm-PTPe is a physiological activator of Src in vivo. Activation is most likely achieved by PTPe dephosphorylating Y527 of Src, thereby opening the intramolecular interaction known to exist between pY527 and the Src SH2 domain and activating the kinase.
2. In the absence of tm-PTPe Src is less active, a fact which causes the tumor cells to alter their morphology and to proliferate more slowly. These aspects of the PTPe-deficient cell phenotype can be rescued by increasing Src activity in the cells artificially.
3. Despite similarities "on paper" in terms of structure and now also in terms of substrate specificity vs. Src, PTPe and the highly related PTPa are not genetically redundant in their activities in Neu-induced mammary tumors. This is evident from the existence of the EKO/Neu phenotype and the changes observed in Src phosphorylation and activity, despite the fact that both types of tumor cells examined - EKO/Neu and Neu - express large and similar amounts of PTPa protein. The molecular mechanism behind

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this effect is not clear. Although the functional relationship between PTPa and Src has not been examined in mammary tumors, it is possible that PTPa is in principle capable of activating Src in these cells, but either cannot sufficiently activate Src on its own or is prevented from doing so. Alternatively, the roles of PTPa and tm-PTPe may not be similar with respect to Src in these tumors. The latter explanation is supported by the finding that PTPa is strongly expressed in all types of mouse mammary tumors examined, while expression of tm-PTPe is strictly limited to tumors initiated by Ras or Neu (Elson and Leder, 1995a). In this respect PTPa is more similar to distantly-related PTPs such as PTPk, PTPH1, and LAR than to PTPe. Furthermore, PTPa is typically expressed in more differentiated human breast tumors (Ardini et al., 2000). PTPa expression might then be associated with reduced tumorigenesis in breast cancer, the opposite of what we describe here for PTPe.

4. Results presented here also suggest that it might be useful in certain clinical circumstances to inhibit Src indirectly via inhibition of PTPe, despite recent development of specific, small-molecule inhibitors of Src. First, expression of PTPe in tumors and in healthy tissues is more restricted than that of Src. One could then limit Src inhibition to specific locations where PTPe is expressed by inhibiting PTPe, without the need to engineer tissue- or cell-specificity into Src inhibitors. Second, PTPe most likely activates Src by dephosphorylating the kinase at Y527, in contrast to small molecule inhibitors of Src, which typically target its ATP binding site (eg. Blake et al., 2000). The effects of inhibiting Src via inhibition of PTPe may then be additive or synergistic with direct inhibition of Src, thereby increasing the efficiency of Src inhibition beyond what is possible using Src inhibitors alone.

3. Searching for substrates of PTPe in mammary tumor cells without prior guessing of substrate identity.

Results outlined above suggested that the "educated guess" approach to identifying substrates of PTPe was extremely useful. However, such guesswork is limited by definition to either what is known or what can be conceived based on known facts; the potential for overlooking other substrates is therefore significant. For this reason we are currently using the D-to-A and C-to-S mutants of tm-PTPe to "fish" for substrates in mammary tumor cells without making any assumptions as to their identity.

Experiments of this type currently focus on cell lines developed from mammary tumors induced by Neu in wild-type mice and in PTPe-deficient mice as described above.

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Substrate-trapping molecules are known to be able to bind substrates and protect them from dephosphorylation by active PTPs present in the same cells, so use of Neu cells, which also express active tm-PTPe, is not considered inappropriate here. Due to difficulties encountered in transforming these cell lines we have developed a series of retroviral expression vectors based on the pBABE retroviral system (Morgenstern et al., 1990). Experiments consist of infecting a given cell line with viral particles which include either the empty pBABE vector (as control), or particles containing wild type, D302A, or C334S tm-PTPe. Following selection with puromycin to eliminate cells not infected, cells are grown for 3-5 passages and then labeled overnight with ^{35}S -labeled methionine. Cells are then lysed in an NP-40 based lysis buffer, after which PTPe is immune-precipitated from the cells by use of anti-FLAG antibodies, targeted at a FLAG epitope tag appended to the C termini of PTPe molecules. Precipitated material is then electrophoresed through 7% or 10% SDS-PAGE gels, blotted onto a membrane, and exposed to film. This approach has the advantage of allowing us to visualize precipitated proteins without encountering difficulties caused sometimes by secondary antibodies used in protein blotting reacting with the precipitating antibodies, if prepared in the same animal species.

Results of a typical experiment of this type are presented in Figure 14. To date, two bands of interest have been detected in these experiments - one representing a protein of approximately 80kDa, and another at approximately 200kDa. p80 seems to bind only wild-type tm-PTPe, not its D-to-A or C-to-S mutants, while p200 binds only WT tm-PTPe. This indicates that both molecules are likely interactors of PTPe; interaction of p200 with PTPe may be dependent on the latter's catalytic activity. Both molecules are therefore not considered at this time likely substrates of PTPe, although their identities and functions are still of obvious interest to us. We are currently examining the identities of both molecules by probing blots obtained in these studies with antibodies against known proteins of the appropriate sizes. So far, we know that the 80kDa band is not PKC isoforms alpha or gamma, and that the 200 kDa molecule is not Neu. Should we, as is reasonable to expect, fail to identify these proteins by this guess-based approach, we will up-scale the immune-precipitation experiments in order to obtain sufficient protein from both bands for protein identification by mass spectrometry or protein sequencing.

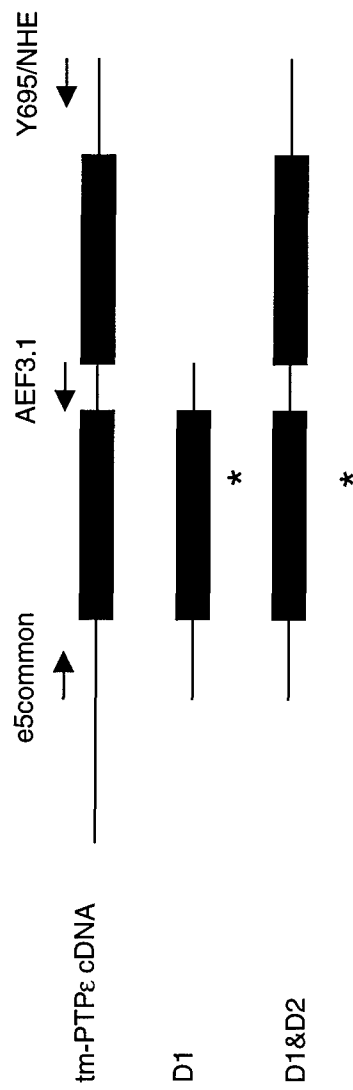


Figure 1: Fragments of tm-PTP ϵ cDNA amplified by PCR for generation of GST-PTP ϵ fusion proteins. Precise sequences of oligonucleotides are given in the text. Black rectangles denote the catalytic domains of PTP ϵ ; asterisks denote locations of the aspartic acid mutated to an alanine in D-to-A mutant versions of these constructs.

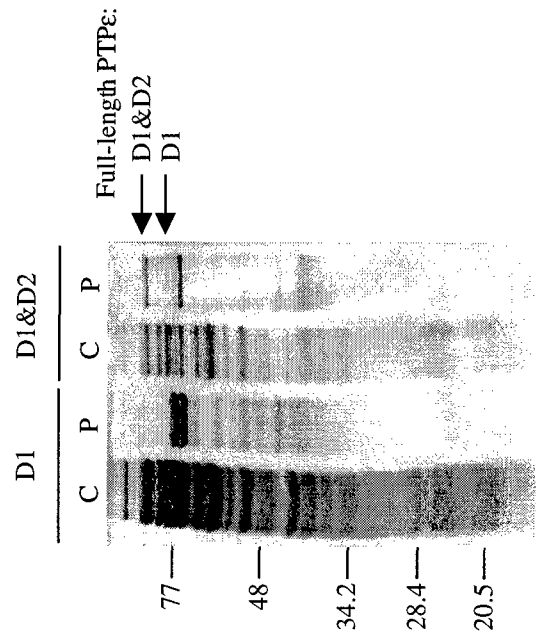


Figure 2: Expression of GST-PTP ϵ fusion proteins. GST fusion proteins of the membrane-proximal catalytic domain of PTP ϵ (D1, construct #308) or of both catalytic domains (D1&D2, construct #304) were constructed as described in the text of the report. Fusion proteins were expressed in BL-21 bacteria and purified using glutathione-agarose beads. Crude bacterial extracts (C) or purified proteins (P) were electrophoresed through a 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Arrows denote location of bands of full length GST-PTP ϵ fusion proteins in the lanes containing pure (P) fusion protein in the D1 or D1&D2 lanes. Numbers on the left indicate molecular size markers (in kDa).

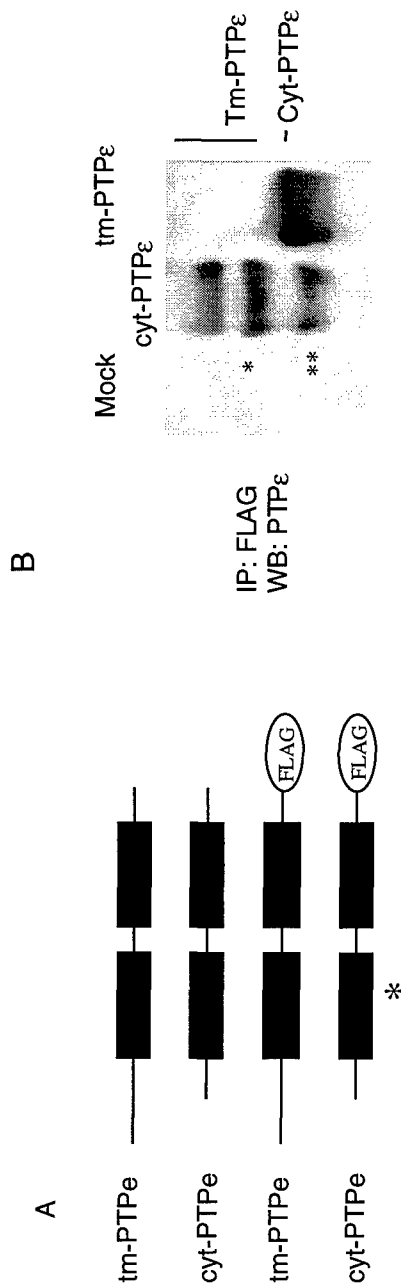


Figure 3: A. Schematic diagram of tm-PTPe and cyt-PTPe cDNAs used in eukaryotic expression studies. Black rectangles denote catalytic domains, ellipses denote FLAG-tag appended to C-termini of some constructs. All cDNAs were cloned into the pCDNA3 expression plasmid. Asterisk marks spot where the D-to-A mutation was inserted. B. Protein blot documenting expression of cDNAs shown in panel A. Proteins were expressed in 293 cells, immune-precipitated using anti-FLAG antibodies, blotted, and probed with an anti-PTPe antibody. Band marked with a single asterisk is unglycosylated tm-PTPe (Elson and Leder, 1995a). Bands marked with two asterisks represent the p67 and p65 isoforms of PTPe (Gil-Henn et al., 2000).

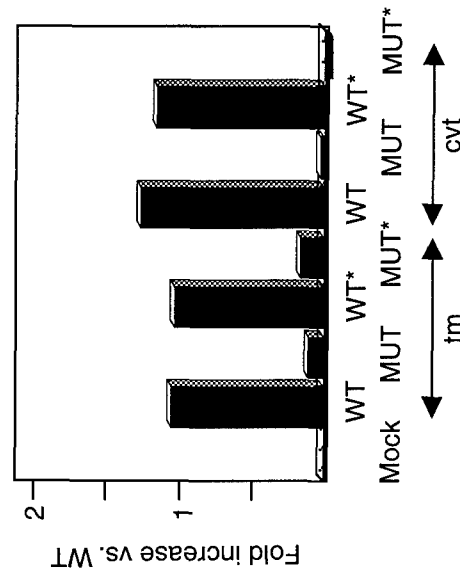


Figure 4: PTPase catalytic activity of wild-type (WT) or D-to-A type mutants (MUT) of tm-PTPe and cyt-PTPe. 293 cells were transfected with the relevant expression vectors, and total PTPase activity, defined as pervanadate-sensitive para-nitrophenylphosphate dephosphorylating activity, was measured. Asterisk denotes FLAG-tagged PTPe. Shown are results of one out of three experiments performed.

Figures 1 through 7 from Peretz et al., 2000, referred to in the text of the report, are located in the copy of the article, which is appended at the end of this report.

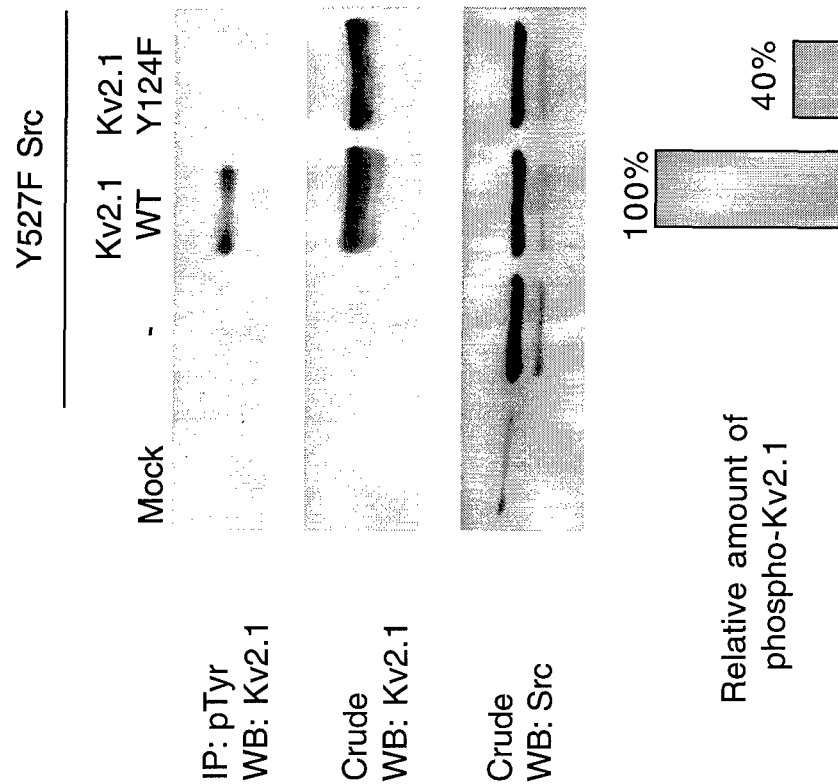


Figure 5: Reduced Src-mediated phosphorylation of Y124F Kv2.1 in comparison with wild-type Kv2.1. 293 cells were transfected with activated (Y527F) Src and wild-type or Y124F Kv2.1 as indicated. Phosphorylated proteins were immunoprecipitated from cell lysates with anti-phosphotyrosine antibodies, and the amount of Kv2.1 in the immunoprecipitates was estimated using anti-Kv2.1 antibodies. Phosphorylation of Y124F Kv2.1, normalized to Kv2.1 expression levels in the cells, was reduced by 60% as compared with wild-type Kv2.1. Shown are results of a single experiment, representative of three performed.

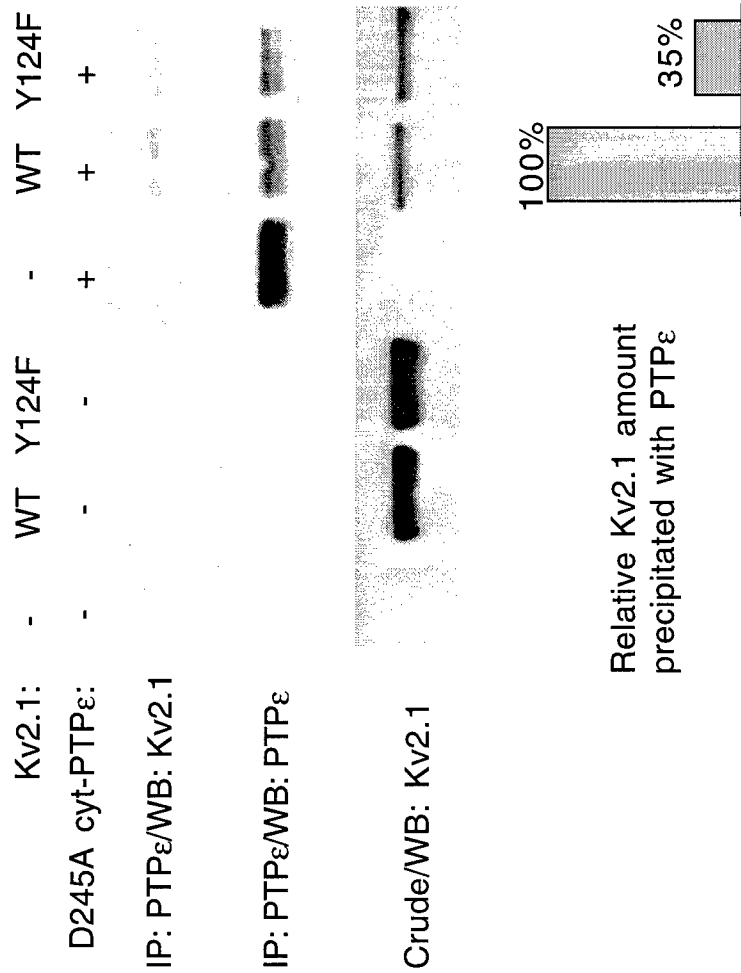


Figure 6: The D245A (substrate-trapping) mutant of cyt-PTP ϵ exhibits reduced binding to Y124F Kv2.1. 293 cells were transfected with activated D245A cyt-PTP ϵ and wild-type or Y124F Kv2.1 as indicated. PTP ϵ was immunoprecipitated from cell lysates with anti-FLAG antibodies, and the amount of Kv2.1 in the immunoprecipitates was estimated using anti-Kv2.1 antibodies. Binding of Y124F Kv2.1, normalized to Kv2.1 expression levels in the cells, was reduced by 65% as compared with wild-type Kv2.1. Experiment shown is representative of four performed.

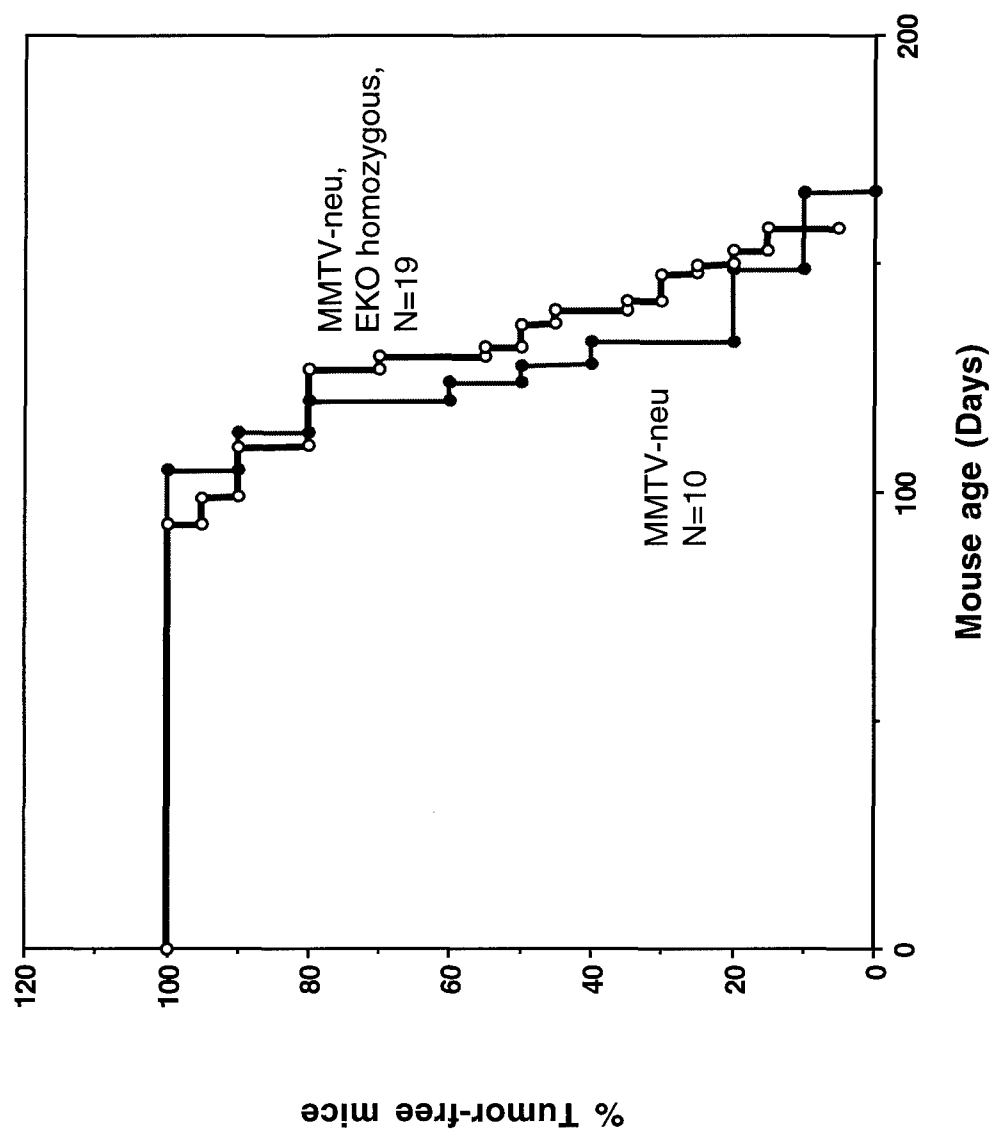
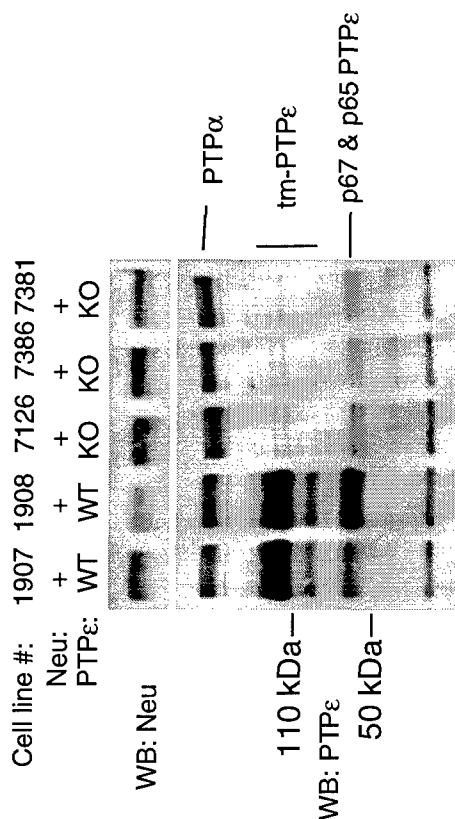


Figure 7: PTPe deficiency does not alter kinetics of tumor formation by the MMTV-activated neu transgene. Female mice of the indicated genotypes were allowed to mate at will and were followed for tumor appearance. Shown is the percentage of tumor-free surviving mice as a function of their age.

A



B

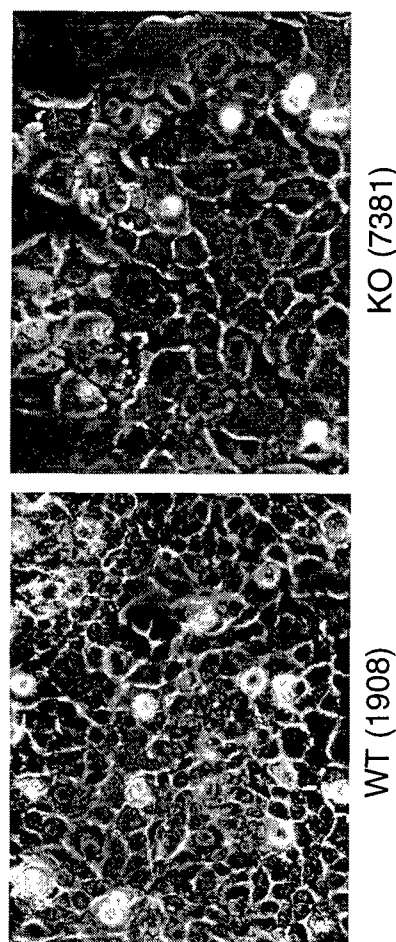


Figure 8: Characteristics of EKO/Neu and Neu mammary tumor cells. A. Protein blot documenting expression levels of Neu, PTPe, and PTPa in cell lines derived from Neu-induced mammary tumors of wild type (WT) and PTPe-deficient (KO) mice. tm-PTPe is either fully glycosylated (heavy band at 105kDa) or non-glycosylated (light band at 85kDa) (Eison and Leder, 1995a). Also noted are the p67 and p65 forms of PTPe (Gil-Henn et al., 2000). Amount of PTPa is likely to be underestimated with respect to PTPe in this figure as the antibody used reacts with PTPe much better. B. Typical morphology of WT (line 1908) and PTPe-deficient (line 7381) tumor cells grown in tissue culture. Phase light microscopy, original magnification 200X.

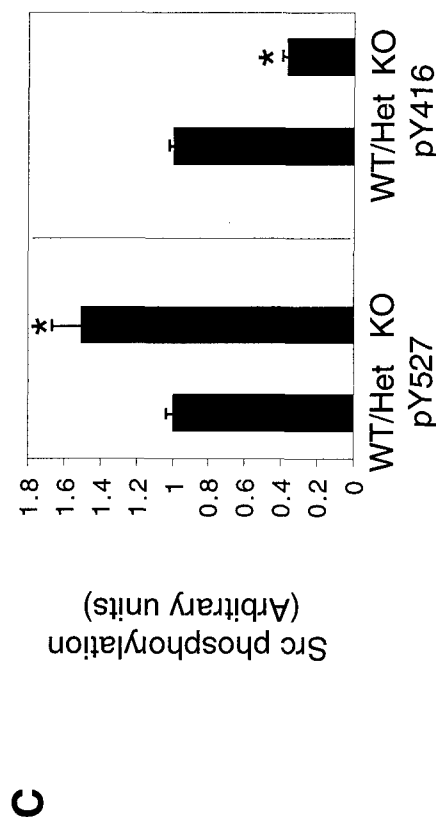
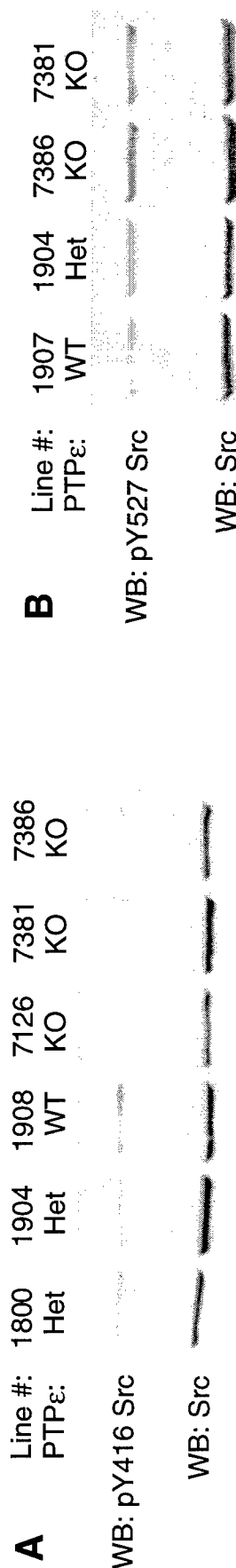


Figure 9: Altered phosphorylation of Src in mammary tumor cells lacking PTPε. Tumor cells examined contain two (WT), one (Het), or no (KO) functional alleles of PTPε. A. Protein blots depicting levels of phospho-pY416 (top panel) or total Src (bottom panel). B. Same as in A, probed for phospho-Y527 Src. C. Bar diagram depicting average levels of phospho-Y416 and phospho-Y517 Src of KO relative to WT/Het cells. n=3-5 in each case. *-p<0.0005 by Student's t-test.

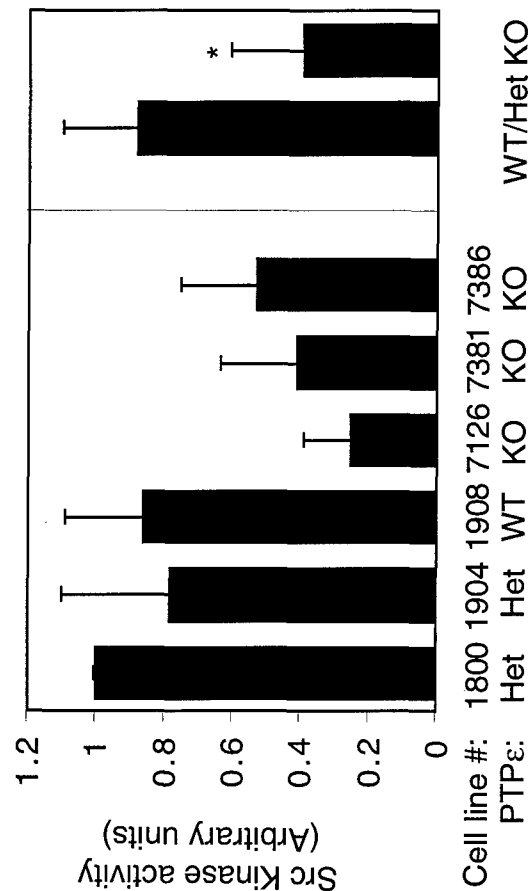


Figure 10: Reduced Src kinase activity in mammary tumor cells lacking PTPε. Bar diagram represents Src kinase activity towards enolase, relative to activity measured in cell line #1800. n=2-3 for each cell line. Left panel represents cumulation of all WT/Het data vs. KO data. *-p=0.0091 by Student's t-test.

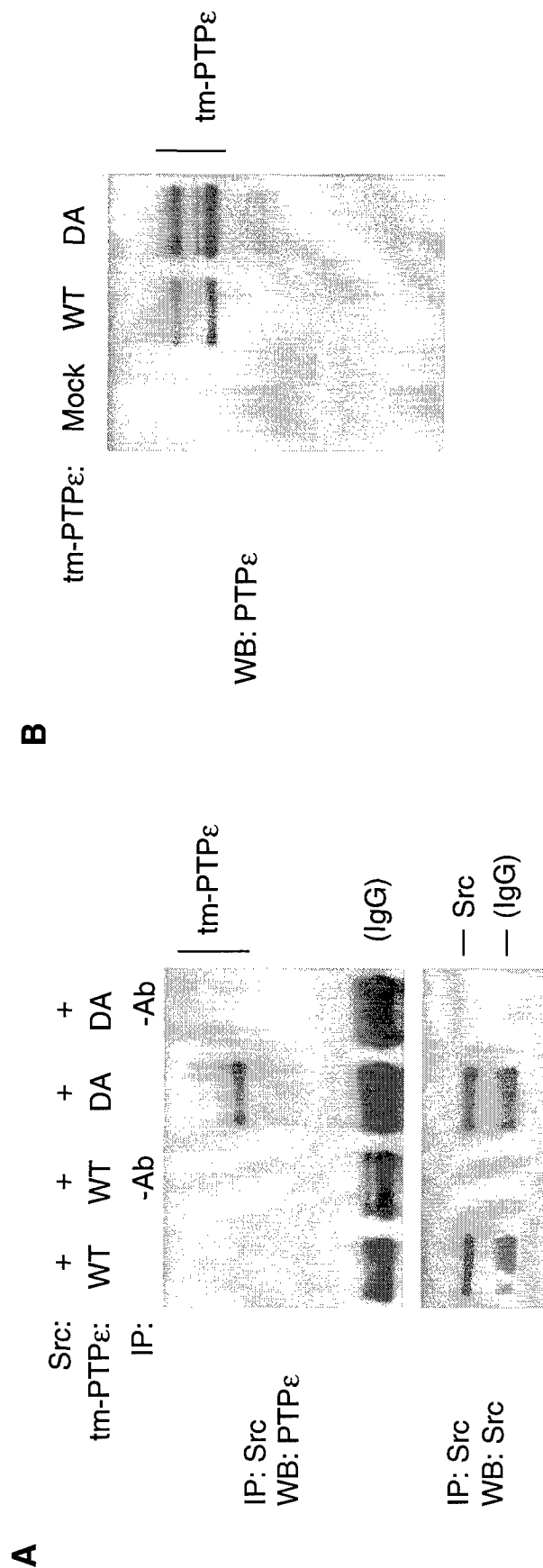


Figure 11: A substrate-trapping mutant of tm-PTPε co-immunoprecipitates Src. A. Src was immune-precipitated from SYF cells, which were transfected with Src and either wild-type (WT) or the substrate-trapping D302A mutant (DA) of tm-PTPε; precipitated material was blotted for presence of associated PTPε (top panel) and for precipitated Src (bottom panel). -Ab: control precipitation reaction performed in absence of primary anti-Src antibody. B. Documentation of expression of WT or D302A tm-PTPε (DA) in transfected cells.

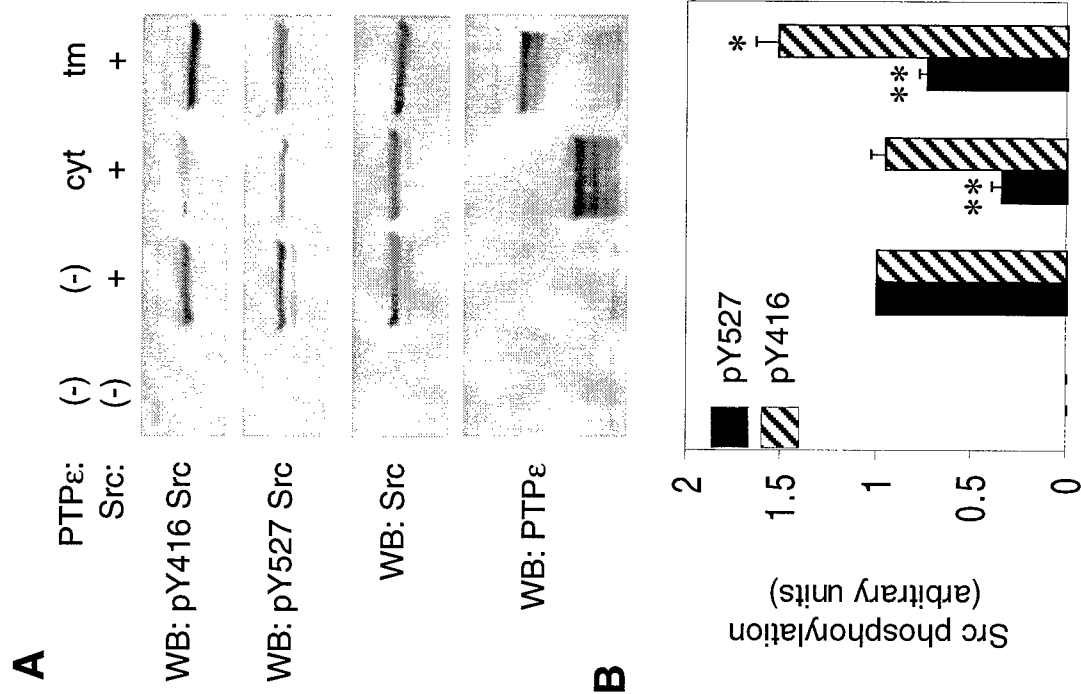


Figure 12: Expression of tm-PTPe or cyt-PTPe affects Src phosphorylation and activity in a manner opposite to that of PTPe deletion. SYF fibroblasts were transfected with c-Src and with either tm-PTPe or cyt-PTPe. A. Protein blots depicting levels of pY416 Src and pY527 Src (top two panels), as well as expression levels of Src (third panel) and PTPe (fourth panel). B. Bar diagram depicting average levels of pY416 and pY527 Src in SYF cells, relative to those measured in cells transfected with Src alone. *-p=0.014; **-p<0.0035 by Student's t-test. C. Bar diagram of Src kinase activity towards enolase in cell lines expressing Src and PTPe, relative to activity in cells expressing Src alone. Similar results were obtained by analyzing Src autophosphorylation (not shown). *-p=0.048; **-p=0.020 by Student's t-test. tm: tm-PTPe. cyt: cyt-PTPe. n=2-5 for each bar.

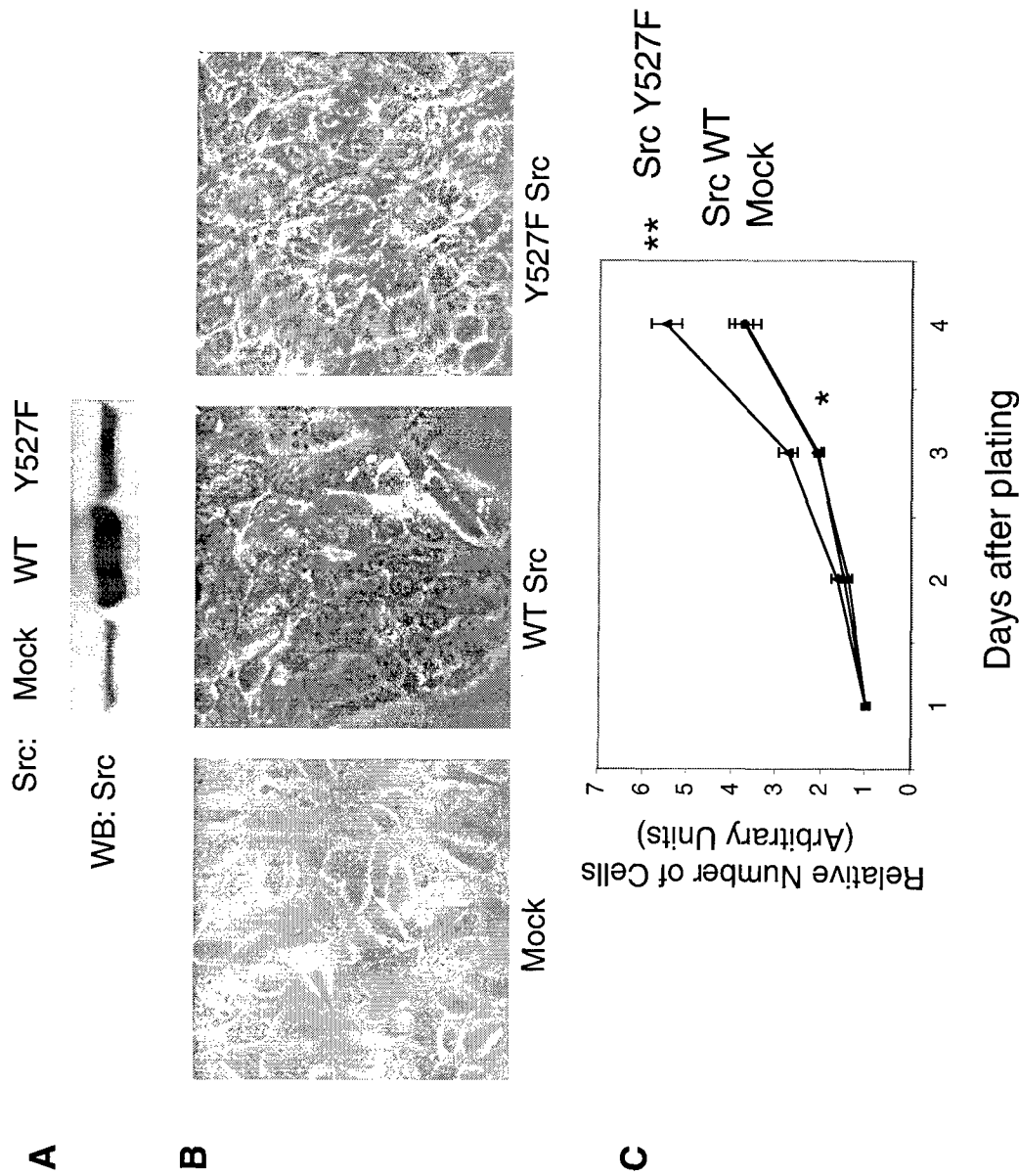


Figure 13: Expression of Src in Neu-induced mammary tumor cells which lack PTPe rescues their morphology and increases their proliferation rate. Mammary tumor cells of PTPe-deficient mice (line 7381) were infected with retroviral vectors containing empty vector (mock), c-Src (WT Src), or constitutively-active (Y527F) Src. Following selection in puromycin and 10-14 days of passaging, cells were analyzed. A. Protein blot depicting relative expression levels of Src in the three cell types. B. Typical morphology of the three cell types. C. Proliferation of the three cell types in culture. Shown is cell number at days 2-4 after plating, relative to that 16 hours after plating. n=6 for each point shown. * $p=0.0395$; ** $p=0.010$ by Student's t-test.

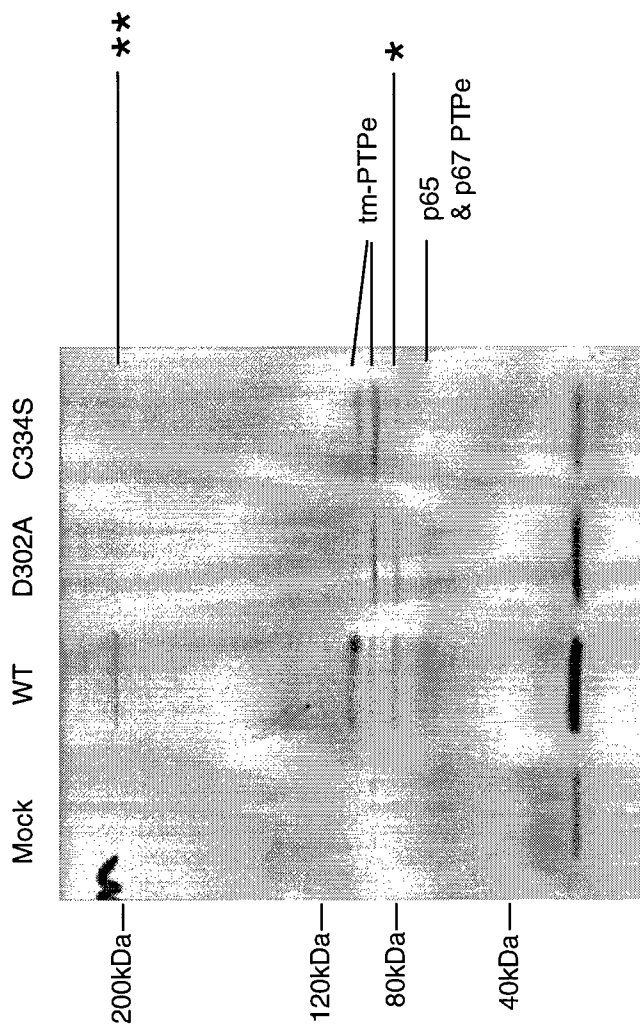


Figure 14: Proteins which co-immunoprecipitate with tm-PTP and with its D302A and C334S substrate-trapping mutants. Neu-induced mouse mammary tumor cells were infected with pBABE empty viral vector (mock) or with pBABE-based retroviral particles containing wild-type tm-PTPe (WT), or its D302A or C334S substrate-trapping mutants. Cells were labeled with ^{35}S -methionine overnight, and then lysed. tm-PTPe was precipitated, and precipitated material was subjected to 7% SDS-PAGE and blotted. Shown is an autoradiogram of a blot from a representative experiment, on which the locations of tm-PTPe and p65 and p67 PTPe are noted. Also noted are locations of unidentified proteins at approximately 80 kDa (*) and 200kDa (**).

Cell growth rates in vitro and in vivo

Cell line	PTPε genotype	Soft agar	Growth rate in plates (relative cell number ±SEM)	Nude mice tumorigenesis (mammary gland, mg±SEM)	Nude mice tumorigenesis (limb, mg±SEM)
1904	Het	+++	3.69 ± 0.18	(-)	(-)
1800	Het	++	5.09 ± 0.23	161.4 ± 15.8	53.5 ± 4.3
1908	WT	+++	4.78 ± 0.20	93.6 ± 23.7	21.7 ± 5.2
7126	KO	+/-	3.27 ± 0.33	58.0 ± 8.0	28.7 ± 7.7
7381	KO	+	2.82 ± 0.35	6.1 ± 4.1	12.1 ± 3.3
7386	KO	+++	3.31 ± 0.28	25.2 ± 8.6	8.9 ± 1.9
All WT/Het			4.52 ± 0.18	127.5 ± 17.0	37.6 ± 5.8
All KO			3.12 ± 0.18 (-31%)	28.1 ± 6.5 (-78%)	17.0 ± 3.6 (-55%)

Table 1: Altered growth properties of cell lines derived from Neu-induced mammary tumors in soft agar, in plates, and in vivo, following injection into nude mice. Tumor cells examined are from mice containing two (WT), one (Het), or no (KO) functional alleles of PTPε. Growth in soft agar is denoted as ranging from poor (+/-) to excellent (+++). Growth rate in culture dishes is presented as the number of cells present 4 days after passaging, relative to the number of cells present 1 day after passaging. N=4-6 for each cell line. Nude mice tumorigenesis results are presented as weight of excised tumor 15 days after injection of 1.5 million cells into site indicated. n=6 for each line at each injection site.

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6. Key Research Accomplishments:

- * Construction of GST fusion proteins of D-to-A substrate-trapping mutants.
- * Construction of D-to-A substrate-trapping mutants of tm-PTP ϵ and of cyt-PTP ϵ , in eukaryotic expression vector form.
- * Demonstration that the eukaryotic reagents behave as expected (catalytically inactive *in vitro* and *in vivo*, bind PTPe substrates as outlined below).
- * Demonstration that alpha-subunits of voltage-gated potassium (Kv) channels are substrates of PTP ϵ . Elucidation of affect of PTPe on channel activity. Elucidation of a complete kinase (Src)-phosphatase loop in regulation of Kv channels in Schwann cells.
- * Identification of Y124 of the Kv2.1 potassium channel as a major site of phosphorylation by Src and dephosphorylation of PTPe.
- * Establishing a series of PTPe-deficient and PTPe-expressing, Neu-induced mouse mammary tumor cell lines.
- * Use of said cell lines to demonstrate that lack of PTPe affects cell morphology and reduces proliferation rates in culture and *in vivo* in a way which argues that PTPe is required for maintaining transformed phenotype.
- * Use of said cell lines and other reagents to shown that Src is a substrate of PTPe. (Lack of PTPe alters phosphorylation and reduces Src activity; overexpression of PTPe induces opposite effects in Src; substrate-trapping mutants of PTPe bind Src).
- * Identification of tm-PTPe as a physiological activator of Src in Neu-induced mouse mammary tumor cells. Basis for suggestion for use of selective inhibitors of PTPe to augment anti-cancer therapy in conduction with anti-Src inhibitors.
- * Determination that PTPe and the closely-related PTPa are not redundant with respect to their activities towards Src in Neu-induced mammary tumors.
- * Preliminary identification of p80 and p200, two protein interactors of tm-PTPe in mouse mammary tumor cells.

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(Refers only to manuscript 3 in manuscript list)

7. Reportable Outcomes:

A. Manuscripts:

1. Peretz, A*, Gil-Henn, H*, Sobko, A., Shinder, V., Attali, B and Elson, A. (2000)
- Hypomyelination and increased activity of voltage-gated potassium channels in mice lacking protein tyrosine phosphatase ϵ . *EMBO J. 19 (15) 4036-4045*
(* - equally contributing first authors).
2. Gil-Henn, H. and Elson, A. - Tyrosine phosphatase Epsilon assists Neu-induced mammary tumorigenesis by activating Src *in vivo*. (*Submitted*).
3. Tiran, Z., Peretz, A., Gil-Henn, H., Attali, B. and Elson, A. - Tyrosine 124 of the delayed-rectifier, voltage-gated potassium channel Kv2.1 is phosphorylated by Src and dephosphorylated by PTP epsilon *in vivo* (*in preparation*).

B. Abstracts/oral presentations at meetings:

1. Concerted activities of protein tyrosine phosphatase epsilon and tyrosine kinases in regulating activity of voltage-gated potassium channels *in vivo*.
Ari Elson, Bernard Attali, Asher Peretz, Alex Sobko, and Hava Gil.
FASEB meeting on kinases and protein phosphorylation. Snowmass, Colorado, USA. July 24-29, 1999. (Poster).
2. Protein tyrosine phosphatase epsilon inactivates voltage-gated potassium channels *in vivo*.
Ari Elson, Bernard Attali, Asher Peretz, Hava Gil, and Alex Sobko.
EMBO-FEBS Workshop on Protein Phosphatases and Protein Dephosphorylation. September 19-24, 1999. De-Panne, Belgium. (Oral presentation).
3. Second International Conference on Signal Transduction (ICST), May 2000, Dubrovnik, Croatia.
PTP Epsilon is required for myelination of peripheral nerves in early post-natal mice.
Ari Elson, Bernard Attali, Asher Peretz, Hava Gil-Henn, Alex Sobko, and Vera Shinder.
(Oral presentation)
4. FASEB meeting on protein phosphorylation. July 2000, Copper Mountain, Colorado, USA.
PTP Epsilon is required for myelination of peripheral nerves in early post-natal mice.
Ari Elson, Bernard Attali, Asher Peretz, Hava Gil-Henn, Alex Sobko, and Vera Shinder.

(Oral presentation)

5. Era of Hope meeting, May 2000, Atlanta, USA.
Tyrosine dephosphorylation and cellular regulation: PTP epsilon down-regulates voltage-gated potassium channels in vivo.
Ari Elson, Asher Peterz, Hava Gil-Henn, Alex Sobko, and Bernard Attali.
(Poster presentation).
6. 11th International Conference on Phosphoproteins and Second Messengers
April 2001, Melbourne, Australia.
PTP Epsilon is required for myelination of peripheral nerves in early post-natal mice.
Ari Elson, Bernard Attali, Asher Peretz, Hava Gil-Henn, Alex Sobko, and Vera Shinder.
7. Tyrosine Phosphorylation and Cell Signaling meeting, Cold Spring Harbor Laboratory, May 2001.
Two contributions to the meeting:
 1. A role for receptor-type PTP Epsilon in promoting Neu-induced mammary tumorigenesis (Oral presentation).
Ari Elson and Hava Gil-Henn.
 2. PTP Epsilon is required for myelination of peripheral nerves in early post-natal mice.
Ari Elson, Bernard Attali, Asher Peretz, Hava Gil-Henn, Alex Sobko, and Vera Shinder (Poster presentation).
8. Hestrin Prize Lecture: Protein tyrosine phosphatase Epsilon - a multi-faceted regulator of physiological events.
Keynote speaker and Hestrin Prize recipient at the annual meeting of the Israeli Society of Biochemistry and Molecular Biology, June 2001.
9. EMBO conference on protein phosphorylation and protein phosphatases (Europhosphatase), July 2001, Marburg, Germany.
Invited lecturer.
A role for receptor-type PTP Epsilon in promoting Neu-induced mammary tumorigenesis (Oral presentation).
Ari Elson and Hava Gil-Henn.
10. Third meeting of the Federation of Israeli Societies for Experimental Biology (FISEB), to be held in Eilat, Israel, in April, 2002.
Invited lecturer.
A role for receptor-type PTP Epsilon in promoting Neu-induced mammary tumorigenesis.
Ari Elson and Hava Gil-Henn.

C. Lectures:

In addition to oral presentations at meetings, lectures on the Kv2.1 and mammary tumorigenesis project were delivered by Dr. Elson during the period of funding at the following venues:

- * Department of Genetics, Hebrew University of Jerusalem, Israel (December, 1999).
- * Department. of Orthopaedics, Yale Medical School (May2000).

- * Department of Genetics, Harvard Medical School (July, 1999; July 2000).
- * Department of Cell Research and Immunology, Tel Aviv University (November, 2000).
- * Department of Pharmacology, Yale Medical School (November 2001).
- * Department of Pharmacology, New York University (November 2001).

D. Cell lines:

A series of 4 mammary tumor cell lines induced by Neu in wild-type mice, as well as three similar lines induced in PTPe-deficient mice has been established.

E. Employment/research opportunities based on this award:

Funds from this award supported work performed by three PhD students in the lab - Ms. Hila Toledano-Katchalski, Ms. Hava Gil-Henn, and Ms. Zohar Tiran. Ms. Toledano-Katchalski has also been receiving a stipend from funds made available to us by this award.

Ms. Toledano-Katchalski and Ms. Gil-Henn will be completing their PhD studies during the first part of 2002.

F. Funding opportunities applied for and received based on findings made in this award:

- * The Israel Science Foundation has awarded us a grant for \$290,000 (over a four year period) to continue studying the Kv channel-PTPe connection.
- * The Minerva Foundation (Munich, Germany) has awarded us a grant of \$150,000 (over three years) to continue studying the PTPe-Src-mammary tumorigenesis connection.

G. Prizes:

Studies outlined here were among the major reasons for Dr. Elson having been awarded the Hestrin Prize for 2001 by the Israeli Society for Biochemistry and Molecular Biology. The Hestrin Prize is awarded annually by the Society to a single young (by Israeli standards) Israeli scientist at the annual meeting of the Society.

Page contains unpublished material, which should be protected

8. Conclusions

The studies described in this report give rise to several main conclusions of both a technical and biological-physiological nature:

Technical conclusions:

1. Our use of C-to-S and especially of D-to-A substrate trapping mutants of PTPe indicates that these mutants can indeed be used to bind physiological substrates of this phosphatase. D-to-A mutants, with which we have had the most experience, bind specific candidate substrates strongly enough such that the PTPe-substrate interactions withstand the rigors of cell lysis, immunoprecipitation and stringent washings.
2. We have had the most success with the "educated guess" approach, i.e., in experiments where we use trapping mutants to either prove or disprove the hypothesis that a given molecule is a substrate of PTPe. As indicated in the body of the report, this approach is inherently limited by whatever information is known from other studies and by one's own imagination. Work of this nature clearly needs complementation by "fishing expeditions", i.e. by attempts to trap substrates without making any assumptions as to their identity. Having established the efficiency of our mutants and working procedures, we are now moving in this direction.

Biological-physiological conclusions:

3. We have shown that the delayed-rectifier, voltage-gated potassium channels Kv2.1 and Kv1.5 are physiological substrates of PTPe *in vivo*. In particular, the non-membrane form of PTPe (cyt-PTPe) dephosphorylates and activates these channels. This has been demonstrated in the ability of PTPe to dephosphorylate and inactivate Kv channels in cultured cells, as well as in the opposite effects (hyperphosphorylation and hyperactivity) lack of PTPe has on Kv channels in Schwann cells of PTPe-deficient mice. Key support for this has come from the ability of a D-to-A substrate-trapping mutant of PTPe to bind Kv2.1.
4. PTPe antagonizes the activities of the Src and Fyn tyrosine kinases towards Kv1.5 and Kv2.1. This defines a complete system by which PTPe counters the activities of these tyrosine kinases towards a set of common substrates, Kv1.5 and Kv2.1.
5. It is reasonable to assume that a regulatory system regulates the activities of the kinases and PTPe vs. Kv channels, such that both arms of this system are not active simultaneously. We are now searching for the identity of this system.

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6. Tyrosine 124 is the amino terminal end of Kv2.1 is a major substrate for both Src and PTPe. The effects of dephosphorylation of Y124 in Kv2.1 are currently being examined.

7. Aberrant dephosphorylation of Kv1.5 and Kv2.1 in Schwann cells of young PTPe-deficient mice correlates with severe transient hypomyelination of sciatic nerve axons. This finding indicates that PTPe fulfills a non-redundant function in Schwann cells of early post-natal mice. The role Kv channels play in Schwann cell function suggests that this finding may be linked to aberrant phosphorylation and activity of these channels in Schwann cells.

8. We have shown that the Src tyrosine kinase is a physiological substrate of PTPe, and that PTPe is a physiological activator of Src. This has been backed up by a series of studies which show that expression of PTPe in cultured cells alters phosphorylation and activates Src, while opposite changes are observed in Neu-induced mammary tumor cells from PTPe-deficient mice. Also, the D-to-A substrate-trapping mutant of PTPe is capable of binding Src.

8. We believe that PTPe dephosphorylates Src primarily at Y527, since this is the major site whose phosphorylation is decreased upon PTPe expression. Furthermore, dephosphorylation of Src at Y527 activates Src, in agreement with the consequences of PTPe activity on the kinase.

9. PTPe and the highly-related PTPa are not redundant with respect to one another in terms of their activity towards Src in Neu-induced mammary tumors. This is an unexpected finding, since both PTPs are known to activate Src. Evidently, overlapping substrate specificity might not always translate into physiological redundancy in vivo.

10. It may therefore be possible to develop and use PTPe inhibitors as indirect inhibitors of Src in the context of breast tumors. An advantage of this approach is that the relatively limited expression pattern of PTPe in organs means inhibiting PTPe would inhibit Src in a very select set of cells and tissues without having to engineer a high degree of specificity in Src inhibitors. Furthermore, small molecule inhibitors of Src and PTPe seem to act on Src by different ways, suggesting that they may act independently or even synergistically, thereby inhibiting Src to greater extents than possible using either approach alone.

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10. Appendices:

Appended is a copy of the manuscript, which describes the Kv-PTPe study. Figures from this manuscript are referred to in the body of the report:

Peretz, A., Gil-Henn, H., Sobko, A., Shinder, V., Attali, B and Elson, A. (2000).

Hypomyelination and increased activity of voltage-gated potassium channels in mice lacking protein tyrosine phosphatase ϵ . *EMBO J.* 19 (15), 4036-4045.

Hypomyelination and increased activity of voltage-gated K⁺ channels in mice lacking protein tyrosine phosphatase ϵ

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A.Peretz and H.Gil-Henn contributed equally to this work

Protein tyrosine phosphatase epsilon (PTP ϵ) is strongly expressed in the nervous system; however, little is known about its physiological role. We report that mice lacking PTP ϵ exhibit hypomyelination of sciatic nerve axons at an early post-natal age. This occurs together with increased activity of delayed-rectifier, voltage-gated potassium (Kv) channels and with hyperphosphorylation of Kv1.5 and Kv2.1 Kv channel α -subunits in sciatic nerve tissue and in primary Schwann cells. PTP ϵ markedly reduces Kv1.5 or Kv2.1 current amplitudes in *Xenopus* oocytes. Kv2.1 associates with a substrate-trapping mutant of PTP ϵ , and PTP ϵ profoundly reduces Src- or Fyn-stimulated Kv2.1 currents and tyrosine phosphorylation in transfected HEK 293 cells. In all, PTP ϵ antagonizes activation of Kv channels by tyrosine kinases *in vivo*, and affects Schwann cell function during a critical period of Schwann cell growth and myelination.

Keywords: myelination/potassium channel/Schwann cells/tyrosine kinase/tyrosine phosphatase

Introduction

Reversible phosphorylation of tyrosine residues in proteins plays a central role in regulation of cellular functions, and is a process controlled by the opposing actions of protein tyrosine kinases (PTKs) and tyrosine phosphatases (PTPases) (Hunter, 1995). Aberrant PTK activity has been linked repeatedly to a wide variety of human diseases, underscoring the pivotal role of accurate tyrosine phosphorylation in physiological processes. PTPases, which are molecularly, biochemically and physiologically distinct from PTKs, have not been studied as extensively as PTKs, although their intimate link to phosphorylation events indicates that PTPases are highly relevant in this respect.

PTPases are a structurally diverse family of transmembranal and cytoplasmic enzymes, of which >70 members have been identified in organisms ranging from viruses to man (Tonks and Neel, 1996). Transmembranal PTPases typically contain two catalytic domains each and are believed to bind extracellular molecules and to participate in signal transduction events (Schaapveld *et al.*, 1997; Zondag and Moolenaar, 1997). Cytoplasmic PTPases

generally contain a single catalytic domain flanked by protein domains, which either regulate the catalytic activity of the molecule or target it to particular regions within the cell (Mauro and Dixon, 1994; Denu and Dixon, 1998). Depending on its context, PTPase activity can enhance or decrease the intensity of transduced signals and is physiologically significant (Tonks and Neel, 1996; Neel and Tonks, 1997; Fischer, 1999). Not surprisingly, several key members of this family have been implicated in control of growth, differentiation and malignant transformation (Tonks and Neel, 1996; Parsons, 1998; den Hertog, 1999).

Protein tyrosine phosphatase epsilon (PTP ϵ ; gene symbol *Ptpre*) is somewhat unique among PTPases in that the single PTP ϵ gene contains two distinct promoters, each of which gives rise to a unique protein product: a transmembranal, receptor-type protein (tm-PTP ϵ) and a second protein, which is predominantly cytoplasmic (cyt-PTP ϵ) (Krueger *et al.*, 1990; Elson and Leder, 1995a,b; Nakamura *et al.*, 1996; Tanuma *et al.*, 1999). Although most of their sequences—including their catalytic domains—are identical, tm- and cyt-PTP ϵ possess unique N-termini that determine their different subcellular localizations and probably distinct physiological roles (Elson and Leder, 1995b). Both forms of PTP ϵ bind Grb2 (Toledano-Katchalski and Elson, 1999); tm-PTP ϵ down-regulates insulin receptor signaling in transfected cells (Moller *et al.*, 1995), has been linked with promotion of mammary tumorigenesis *in vivo* (Elson and Leder, 1995a; Elson, 1999) and may play a role in regulating osteoclast function (Schmidt *et al.*, 1996).

It is reasonable to expect that additional physiological roles of PTP ϵ will involve molecules whose activity is regulated by tyrosine phosphorylation. Prominent among these are K⁺ channels, as several studies have shown that these are substrates of protein kinase activities (Levitani, 1994, 1999; Siegelbaum, 1994; Jonas and Kaczmarek, 1996). Heterologous expression studies indicate that the activity of several members of the Kv1 family of delayed-rectifier K⁺ (Kv) channels is altered following phosphorylation by non-receptor and receptor tyrosine kinases (Huang *et al.*, 1993; Timpe and Fantl, 1994; Holmes *et al.*, 1996; Bowlby *et al.*, 1997; Fadool *et al.*, 1997; Tsai *et al.*, 1997; Wang, 1999). *In vivo*, phosphorylation of K⁺ channels by PTKs can either activate or down-regulate channel activity. Along these lines, activated insulin receptor increases delayed-rectifier K⁺ currents in *Aplysia* bag cell neurons (Jonas *et al.*, 1996), while in the Jurkat human T-cell line, p56^{lck}-mediated phosphorylation of the Kv1.3 potassium channels down-regulates voltage-sensitive K⁺ currents (Szabo *et al.*, 1996). Recently, we showed that the delayed-rectifier *I_K* is markedly up-regulated following intracellular application of recombinant Fyn tyrosine kinase to mouse Schwann

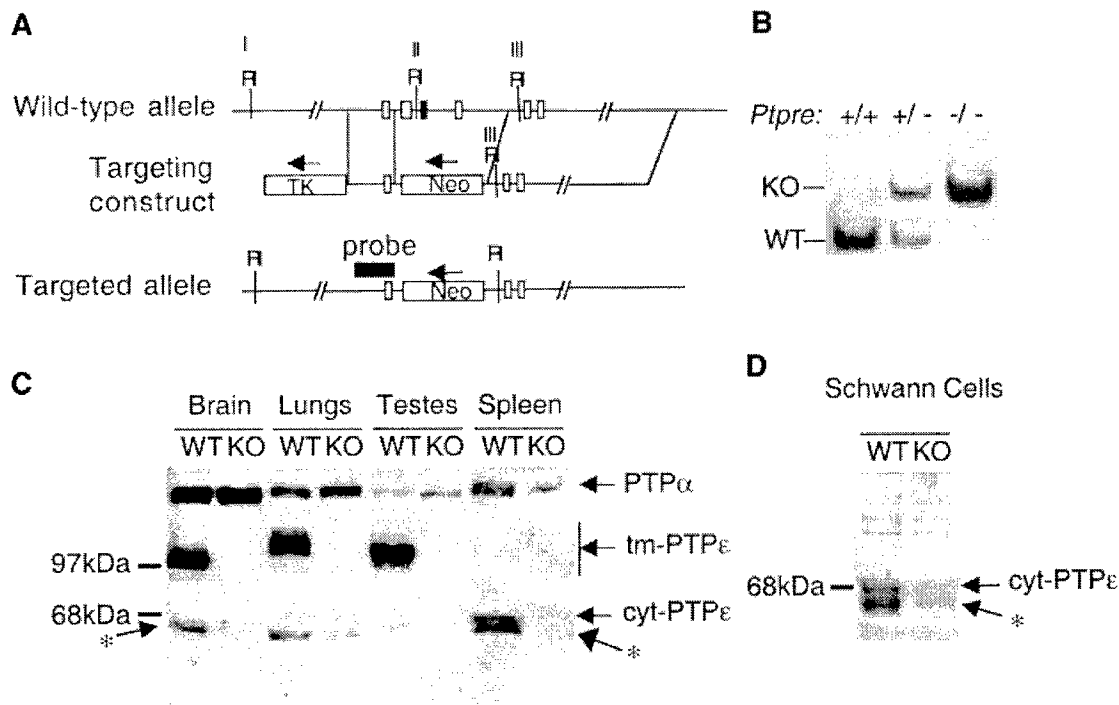


Fig. 1. Generation and characterization of *Ptpre*^{-/-} mice. (A) Schematic representations of the region of the mouse *Ptpre* gene chosen for targeting (top), targeting construct (middle) and recombinant allele (bottom). Homologous recombination removed three exons (rectangles), including one (black rectangle) containing C277, the catalytic cysteine of the membrane-proximal catalytic domain. Flanking regions are 1.2 (5') and 10 kb (3') in length. RI-EcoRI sites in the targeted region. (B) DNA blot analysis of genomic DNA from *Ptpre*^{+/+}, *Ptpre*^{+/-} and *Ptpre*^{-/-} mice. Genomic DNA was digested with *Eco*RI and probed with the genomic fragment indicated in (A). Fragments originating in the wild-type (WT; 10.0 kb) and targeted (KO; 11.8 kb) alleles, respectively, are indicated. (C) Protein blot documenting lack of expression of PTPε in *Ptpre*^{-/-} mice. Protein extracts from the indicated organs of WT or KO mice were analyzed using an anti-PTPε antibody (Elson and Leder, 1995a). Antibody also cross-reacts with PTPα as indicated in the figure. Variations in size of tm-PTPε from brain, lungs and testes are due to tissue-specific glycosylation (Elson and Leder, 1995a). Note the presence in several lanes of PTPε-related bands migrating at ~68 kDa, (asterisk), slightly faster than cyt-PTPε. (D) Protein blot analysis as in (C), documenting expression levels of cyt-PTPε in primary WT or KO Schwann cells derived from 3- to 5-day-old pups.

cells, and is down-regulated upon exposure to tyrosine kinase inhibitors (Sobko *et al.*, 1998a; Peretz *et al.*, 1999). In contrast, very little is known about the *in vivo* regulation of K⁺ channel activity by PTPases, although a recent study showed that *in vitro* expression of PTPα correlates with activation of Kv1.2 channels (Tsai *et al.*, 1999).

The intimate connection between regulation of Kv channels and PTKs prompted us to examine whether PTPε is involved in this process *in vivo*. For this purpose we examined Kv channel function in gene-targeted mice that lack PTPε (*Ptpre*^{-/-} mice). Lack of PTPε causes hypomyelination of sciatic nerve axons in newborn mice, together with increased Kv channel activity and hyperphosphorylation of the Kv1.5 and Kv2.1 α-subunits in cultured primary Schwann cells and sciatic nerve tissue. *In vitro* studies show that Kv2.1 interacts with the active site of PTPε, and that active PTPε can down-regulate Kv1.5 and Kv2.1 channel activity and phosphorylation. Our results indicate that a unique role of PTPε *in vivo* is to down-regulate Kv channel activity in post-natal mice, thereby antagonizing channel activation by tyrosine kinases such as Src and Fyn. PTPε is then part of the finely tuned molecular mechanism, which regulates Kv channel activity during a critical period of Schwann cell development and myelination of peripheral nerves.

Results

Ptpre-deficient mice exhibit peripheral myelination abnormalities

The *Ptpre* gene was targeted by replacing the genomic sequence corresponding to amino acid residues 262–411 of mature tm-PTPε with a selectable neomycin resistance gene (Figure 1A). This region is common to both tm- and cyt-PTPε (Elson and Leder, 1995b), and was chosen so as to disrupt both forms of the enzyme. Following electroporation and selection, clones of *Ptpre*^{+/+} embryonic stem (ES) cells were used to generate chimeric mice and subsequently *Ptpre*^{+/-} and *Ptpre*^{-/-} mice (Figure 1B). tm-PTPε is known to be expressed mainly in brain, as well as in lungs and testes, while cyt-PTPε is expressed mainly in the hematopoietic system (Elson and Leder, 1995b; Mukouyama *et al.*, 1997); protein blot analysis indicated that *Ptpre*^{-/-} mice do not express tm- or cyt-PTPε proteins in these tissues (Figure 1C). As the antibody used was raised against a peptide located upstream of the targeting site (Elson and Leder, 1995a), it would have allowed the detection of possible truncated protein products; none were detected. The antibody cross-reacts with the closely related PTPα; no compensatory changes in the amounts of PTPα protein were detected in the organs examined (Figure 1C).

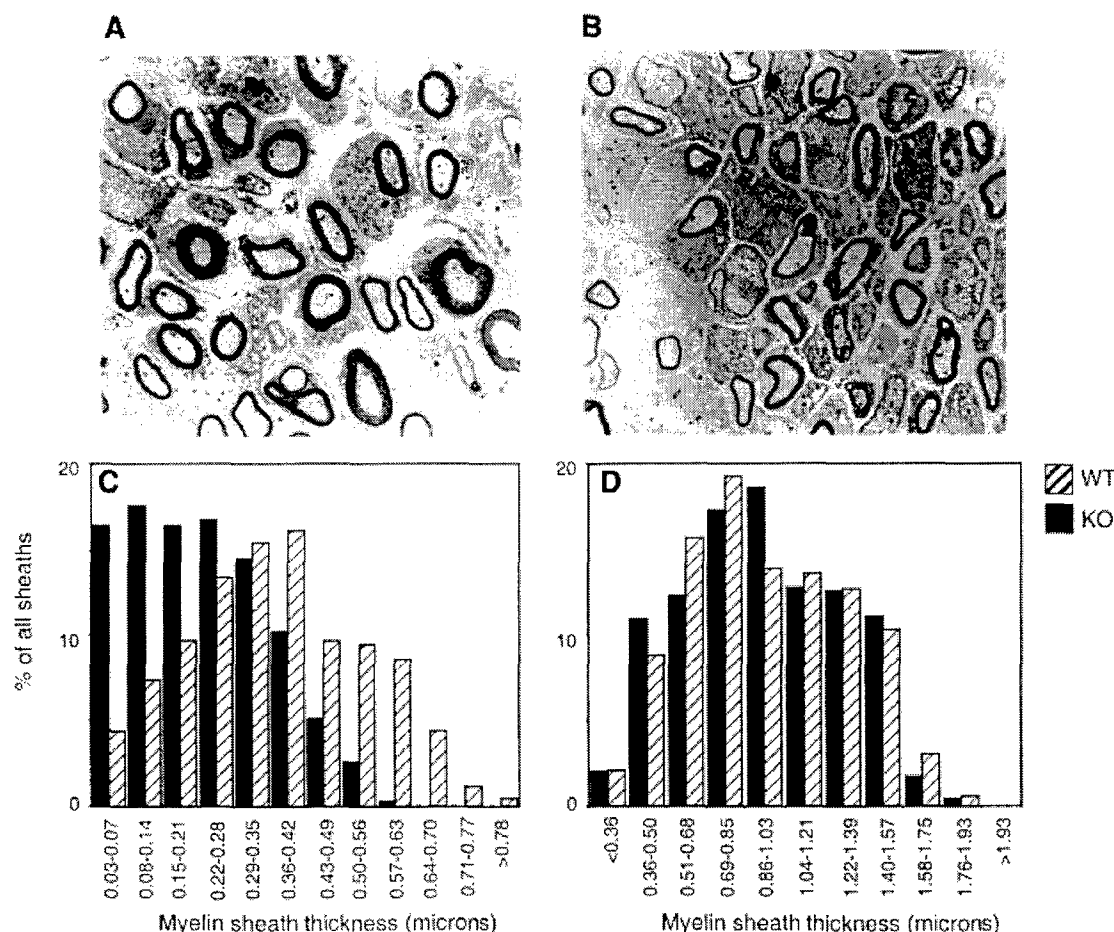


Fig. 2. Reduced myelination of axons in sciatic nerves of 5-day-old *Ptpre*^{-/-} mice. Cross-section of sciatic nerve of WT (A) or *Ptpre*^{-/-} (B) mice. Magnification 3400 \times . (C) Distribution of widths of myelin sheaths of axons in sciatic nerves of 5-day-old WT (hatched bars) and KO (black bars) mice. Mean sheath thicknesses (in μ m, \pm SEM) were: *Ptpre*^{-/-}: 0.23 ± 0.05 , $n = 456$; wild type: 0.37 ± 0.06 , $n = 526$ ($p < 0.0001$ by the Mann-Whitney test). (D) As in (C), data from adult, 8-month-old male mice. Mean sheath thicknesses (in μ m, \pm SEM) were: *Ptpre*^{-/-}: 0.92 ± 0.02 , $n = 448$; wild type: 0.94 ± 0.02 , $n = 331$.

Ptpre^{-/-} mice were born at the expected Mendelian ratios from matings of *Ptpre*^{+/-} mice and were normal in appearance. However, detailed examination of early post-natal (3- to 5-day-old) *Ptpre*^{-/-} mice revealed myelination defects in the peripheral nervous system. Specifically, myelin sheaths surrounding axons in sciatic nerve fibers of *Ptpre*^{-/-} mice were significantly thinner than in WT (wild-type *Ptpre*^{+/+}) mice (Figure 2A and C). Thinning of myelin sheaths was most evident in the nearly total disappearance of the very thickest sheaths ($>0.6 \mu$ m) from *Ptpre*^{-/-} sciatic nerves, together with a 2.6-fold increase in the fraction of thinly myelinated axons ($<0.14 \mu$ m). Interestingly, myelination of sciatic nerve axons in adult (8 months) *Ptpre*^{-/-} mice was normal (Figure 2D). These results suggest that PTPe performs a unique role in Schwann cells during early myelinogenesis, an important developmental period when Schwann cells cease dividing and differentiate. In adult mice, on the other hand, this function is either not required or can be performed by other PTPases.

Up-regulation of Kv channel activity and phosphorylation in PTPe-deficient Schwann cells

Recent evidence indicates that phosphorylation of Kv channels by Src family kinases could be important for Schwann cell development and peripheral myelinogenesis. In particular, the Fyn tyrosine kinase constitutively activates Kv channels in proliferating Schwann cells (Sobko et al., 1998a; Peretz et al., 1999). After birth, a developmental decrease in Fyn kinase and Kv channel activities may contribute to the exit of Schwann cells from the cell cycle and onset of myelination (Sobko et al., 1998a,b). As tyrosine phosphatases generically counter activities of tyrosine kinases, we sought to determine whether lack of PTPe could modulate Kv channels and Schwann cell function *in vivo*. WT Schwann cells express cyt-PTPe; the enzyme is missing from cells of *Ptpre*^{-/-} mice (Figure 1D). The activity of Kv channels was monitored by recording K⁺ currents in Schwann cells from WT and *Ptpre*^{-/-} mice using the whole-cell configuration

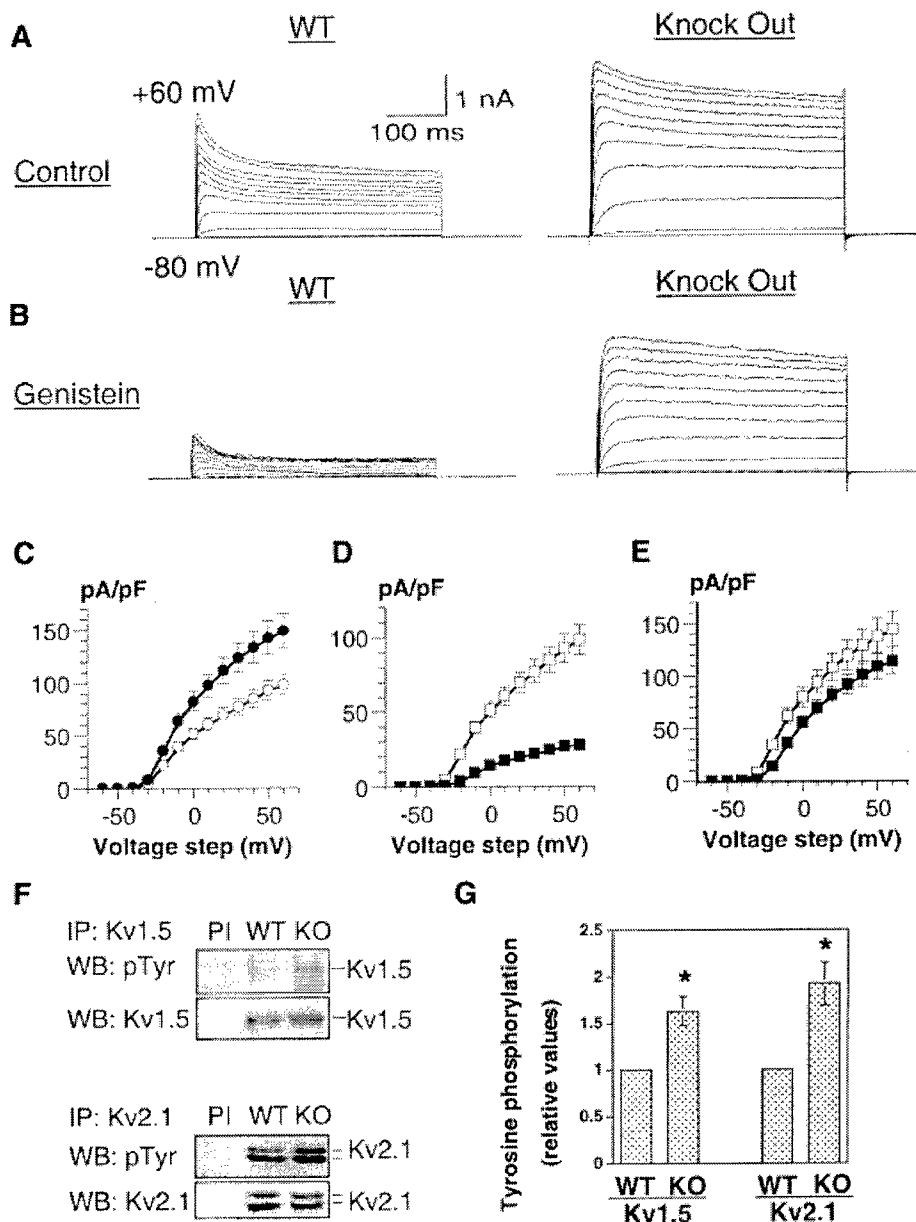


Fig. 3. Characteristics of voltage-gated K⁺ currents in Schwann cells from WT and *Ptpre*^{-/-} mice and tyrosine phosphorylation of delayed-rectifier Kv channel α -subunits. (A and B) Whole-cell K⁺ currents recorded from WT and *Ptpre*^{-/-} Schwann cells, before (A) and after (B) application of 100 μ M genistein for 20 min. Cells were stepped from a holding potential of -80 mV to +60 mV in +10 mV increments for 400 ms pulse duration. (C) The K⁺ current density (pA/pF) of WT ($n = 35$, open circles) and *Ptpre*^{-/-} ($n = 40$, solid circles) Schwann cells was plotted against voltage steps (mV). *Ptpre*^{-/-} Schwann cells exhibit a significantly higher current density when compared with WT cells ($p < 0.01$). (D and E) Current density-voltage relationships ($n = 28$) of WT (D) and *Ptpre*^{-/-} (E) Schwann cells, before (open squares) and following (solid squares) exposure to 100 μ M genistein for 20 min. (F) Representative experiment showing hyperphosphorylation of Kv1.5 and Kv2.1 in Schwann cells of *Ptpre*^{-/-} mice as compared with WT cells. Total protein was immunoprecipitated with anti-Kv1.5 or anti-Kv2.1 antibodies followed by blotting and probing with anti-phosphotyrosine antibodies. Blots were then stripped and re-probed for Kv1.5 or Kv2.1; similar results were obtained for Kv2.1 in sciatic nerve tissue (not shown). (G) Quantification of tyrosine phosphorylation of Kv1.5 and Kv2.1 in WT and *Ptpre*^{-/-} Schwann cells. Phosphorylation of Kv1.5 is increased by $62.5 \pm 15.4\%$ ($n = 4$), while that of Kv2.1 is increased by $91.3 \pm 22.8\%$ ($n = 6$); an asterisk indicates statistical significance ($p < 0.01$) in both cases.

of the patch-clamp technique. These measurements revealed that the maximal K⁺ current density of *Ptpre*^{-/-} Schwann cells increased significantly by 52% when compared with WT cells [from 98.02 ± 9.86 pA/pF (WT) to 149.13 ± 16.11 pA/pF (*Ptpre*^{-/-}) at +60 mV; Figure 3A and C]. No difference was found in the voltage-dependence characteristics of channel activation between

Ptpre^{-/-} and WT Schwann cells (data not shown), suggesting that PTPε could affect the number of functional channels or the unitary channel conductance. In parallel, two Kv α -subunits, Kv1.5 and Kv2.1, were significantly hyperphosphorylated in *Ptpre*^{-/-} Schwann cells (Figure 3F and G) and in sciatic nerve tissue (data not shown). In *Ptpre*^{-/-} Schwann cells, the Kv1.5 and Kv2.1 phospho-

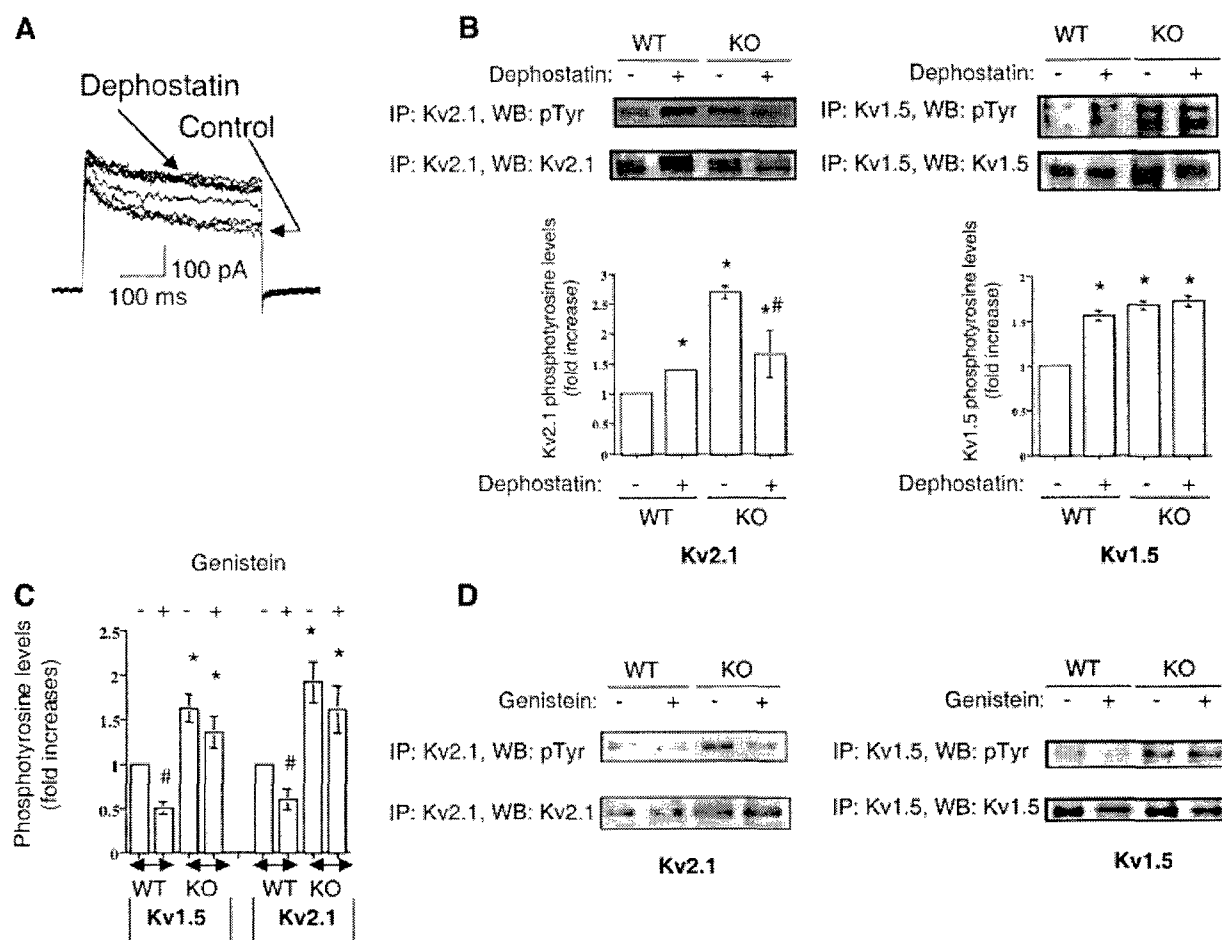


Fig. 4. Effects of dephostatin and genistein on tyrosine phosphorylation of Kv1.5 and Kv2.1 channel α -subunits. **(A)** Train of current traces recorded from the same WT *Ptpr ϵ ^{+/+}* Schwann cell, before and after 50 μ M dephostatin treatment (20 min). The cell was stepped every minute from a holding potential of -80 mV to $+40$ mV (400 ms). **(B)** Effects of dephostatin (50 μ M, 20 min) on tyrosine phosphorylation of Kv1.5 and Kv2.1 α -subunits in WT *Ptpr ϵ ^{+/+}* and KO *Ptpr ϵ ^{-/-}* Schwann cells. Upper panel, representative experiment showing that dephostatin produces an increase in tyrosine phosphorylation of Kv1.5 and Kv2.1 channel α -subunits in WT but not in KO Schwann cells. Lower panel, quantification of tyrosine phosphorylation of Kv1.5 and Kv2.1 in WT and KO Schwann cells before and after dephostatin treatment. Dephostatin increases Kv1.5 and Kv2.1 tyrosine phosphorylation by $56 \pm 6\%$ and $39 \pm 2\%$, respectively ($n = 3$, $p < 0.05$). An asterisk indicates a statistically higher value when compared with WT untreated Schwann cells ($p < 0.05$). Dephostatin significantly reduces, by $38 \pm 14\%$, the phosphotyrosine levels of Kv2.1 in KO Schwann cells ($n = 3$, $\#p < 0.05$). Total proteins were immunoprecipitated, blotted and probed as in Figure 3F. **(C)** Quantification of the effects of genistein (100 μ M, 20 min) on tyrosine phosphorylation of Kv1.5 and Kv2.1 in WT and KO Schwann cells. Genistein reduces by $50 \pm 7\%$ and $40 \pm 13\%$ the tyrosine phosphorylation levels of Kv1.5 and Kv2.1, respectively ($n = 3$, $\#p < 0.05$). An asterisk indicates a statistically higher value when compared with WT untreated Schwann cells ($p < 0.05$). **(D)** Representative experiment showing that genistein decreases significantly the tyrosine phosphorylation of Kv1.5 and Kv2.1 channel α -subunits in WT. The inhibitory effect of genistein is much weaker, if present at all, in KO Schwann cells. Total proteins were immunoprecipitated, blotted and probed as in Figure 3F.

tyrosine levels were up-regulated by $62.5 \pm 15.4\%$ ($n = 4$, $p < 0.01$) and $91.3 \pm 22.8\%$ ($n = 6$, $p < 0.01$), respectively, when compared with WT cells (Figure 3F and G). The data indicate that cyt-PTPase activity normally leads to dephosphorylation of Kv α -subunits and to down-regulation of Kv channel activity *in vivo*. The data also support the correlation observed between hyperphosphorylation of Kv channel α -subunits and increased channel activity (Sobko et al., 1998a; Peretz et al., 1999).

Up-regulation of Kv currents observed in *Ptpr ϵ ^{-/-}* Schwann cells agrees with results obtained in WT Schwann cells following broad-spectrum inhibition of tyrosine phosphatases or tyrosine kinases. Dephostatin, an inhibitor of tyrosine phosphatases, causes up-regulation of

Kv currents and hyperphosphorylation of Kv1.5 and Kv2.1 channel α -subunits in WT Schwann cells (Figure 4A and B). Interestingly, dephostatin-mediated up-regulation of Kv1.5 and Kv2.1 tyrosine phosphorylation is lost in *Ptpr ϵ ^{-/-}* Schwann cells (Figure 4A and B). Dephostatin can eventually reduce the phosphotyrosine levels of Kv2.1 in cells from knockout animals, probably by inhibiting other tyrosine phosphatases, which modulate Kv2.1 channels in a manner different from that of cyt-PTPase (Figure 4B). In agreement with this result, the tyrosine kinase inhibitor genistein exerts the opposite effect and down-regulates Kv channel activity in WT Schwann cells (Sobko et al., 1998a; Peretz et al., 1999). Thus, in WT cells, genistein down-regulates tyrosine phosphorylation levels of Kv1.5

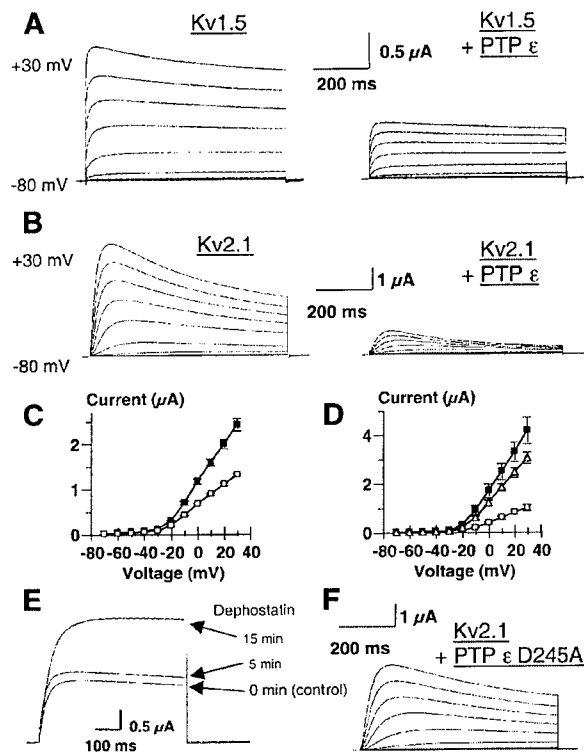


Fig. 5. Effects of cyt-PTPε and D245A cyt-PTPε substrate-trapping mutant on Kv2.1 and Kv1.5 K⁺ currents expressed in *Xenopus* oocytes. (A) Macroscopic K⁺ currents recorded from oocytes microinjected with Kv1.5 (left) or Kv1.5 and cyt-PTPε cRNAs (right) were elicited by depolarizing pulses (700 ms) from a -80 mV holding potential to +30 mV in 10 mV increments. (B) Macroscopic K⁺ currents recorded as in (A), from oocytes microinjected with Kv2.1 (left) and cyt-PTPε cRNAs (right). (C) Current-voltage relationships of Kv1.5 ($n = 14$, solid squares) and Kv1.5 plus cyt-PTPε-mediated K⁺ currents ($n = 16$, open squares). cyt-PTPε decreases Kv1.5 currents significantly ($p < 0.01$). (D) Current-voltage relationships of Kv2.1 ($n = 15$, solid squares), Kv2.1 plus cyt-PTPε ($n = 16$, open squares) and Kv2.1 plus D245A PTPε-mediated K⁺ currents ($n = 14$, open triangles). cyt-PTPε decreases Kv2.1 currents significantly ($p < 0.01$). (E) Representative train of current traces recorded from oocytes microinjected with Kv2.1 and cyt-PTPε cRNAs, before and after 50 μM dephostatin treatment. The oocyte was stepped from a holding potential of -80 mV to +20 mV (600 ms). (F) Macroscopic K⁺ currents recorded as in (A), from oocytes microinjected with Kv2.1 and the D245A PTPε mutant.

and Kv2.1 channel α-subunits by $50 \pm 7\%$ ($n = 3$, $p < 0.01$) and $40 \pm 12\%$ ($n = 3$, $p < 0.05$), respectively (Figure 4C and D). Tyrosine phosphorylation of Kv channel α-subunits is then finely regulated by opposing activities of tyrosine kinases and tyrosine phosphatases (Jonas and Kaczmarek, 1996; Levitan, 1999). These experiments also link cyt-PTPε in particular to down-regulation of Kv currents, as the effects of genistein and dephostatin are markedly altered in *Ptpre*^{-/-} Schwann cells. Genistein (100 μM) reduces Kv current density by 62% in WT Schwann cells, compared with only a 27% reduction in *Ptpre*^{-/-} cells (Figure 3A, B, D and E). In line with this result, genistein does not alter significantly the Kv1.5 or Kv2.1 phosphotyrosine steady-state levels in *Ptpre*^{-/-} Schwann cells (Figure 4C and D). Similar results were obtained with herbimycin A (data not shown). The inhibitory effect of genistein or herbimycin A on Kv

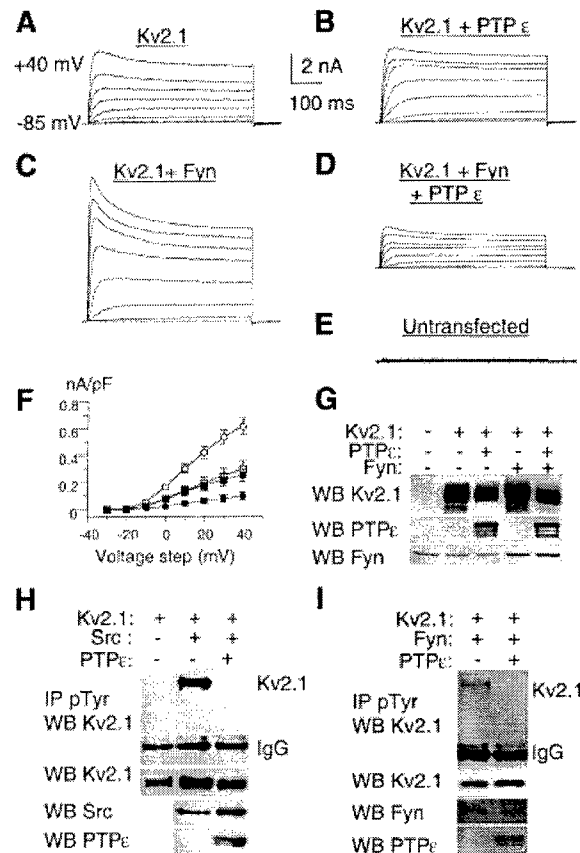


Fig. 6. Effects of cyt-PTPε, activated Fyn and Src kinases on Kv2.1 tyrosine phosphorylation and K⁺ currents expressed in HEK 293 cells. (A-E) Representative whole-cell K⁺ currents from cells transfected with Kv2.1 (A), Kv2.1 + cyt-PTPε (B), Kv2.1 + activated Fyn kinase (C), Kv2.1 + activated Fyn kinase + cyt-PTPε (D) cDNAs, respectively, as well as from an untransfected cell (E). Currents were elicited by depolarizing pulses (500 ms) from a -85 mV holding potential to +40 mV in 20 mV increments. The scale bars are the same for A-E. (F) Current-voltage relationships of K⁺ currents produced by cells transfected with Kv2.1 ($n = 15$, open squares), Kv2.1 + cyt-PTPε ($n = 5$, solid squares), Kv2.1 + activated Fyn kinase ($n = 12$, open circles) and Kv2.1 + activated Fyn kinase + cyt-PTPε ($n = 14$, filled circles) cDNAs. (G) Documentation of expression of Kv2.1, cyt-PTPε and Fyn in HEK 293 cells used for electrophysiological studies presented in (A-E). Cells were transfected with plasmids for the indicated proteins, and protein blots prepared from their extracts were probed with the indicated antibodies. (H and I) Cyt-PTPε reduces, respectively, Src- (H) and Fyn-mediated (I) phosphorylation of Kv2.1 in transfected cells. HEK 293 cells were transiently transfected with Kv2.1, activated Fyn (Y531F), activated Src (Y527F), cyt-PTPε cDNAs, or combinations thereof. Tyrosine-phosphorylated proteins were immunoprecipitated and the amount of Kv2.1 in the precipitate was analyzed by protein blotting (top panel). The second, third and fourth panels document expression of Kv2.1, Fyn/Src and cyt-PTPε, respectively, in the transfected cells.

channel activity is then partly countered by lack of cyt-PTPε activity in *Ptpre*^{-/-} Schwann cells.

cyt-PTPε down-regulates Kv channel activity and phosphorylation in vitro

To investigate direct modulation of Kv channels by cyt-PTPε further, we co-expressed Kv1.5 or Kv2.1 with cyt-PTPε in *Xenopus* oocytes and HEK 293 cells (Figures 5 and 6). In *Xenopus* oocytes, co-expression of Kv1.5 or

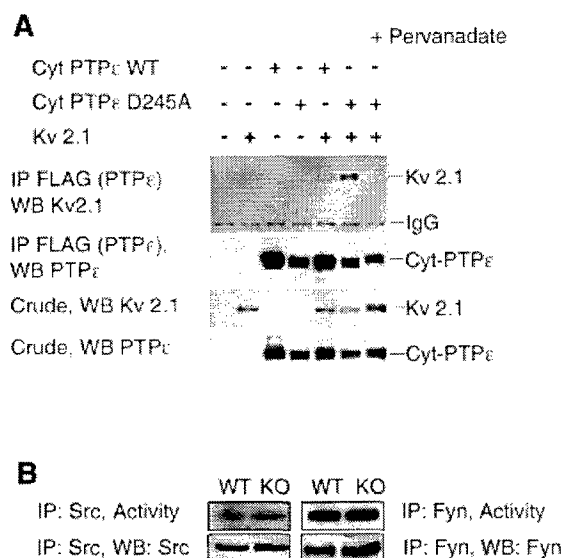


Fig. 7. (A) Kv2.1 preferentially associates with the D245A cyt-PTP ϵ substrate-trapping mutant. HEK 293 cells were transiently transfected with wild-type cyt-PTP ϵ , D245A cyt-PTP ϵ , Kv2.1, or combinations thereof. Following lysis, PTP ϵ was immunoprecipitated via its attached FLAG tag; associated Kv2.1 was detected by protein blot analysis of immunoprecipitates (top panel). The second, third and fourth panels document expression of cyt-PTP ϵ and Kv2.1 and immunoprecipitation of cyt-PTP ϵ in the various transfections. The presence of pervanadate (right-most lane) somewhat reduces the electrophoretic mobility of some of the proteins examined, most likely by more effectively reducing their dephosphorylation by phosphatases present in cell lysates during precipitation. (B) Expression and activities of Src and Fyn are unaltered in *Ptpre*^{-/-} Schwann cells. Total protein from primary Schwann cells of 3- to 5-day-old mice were immunoprecipitated with anti-Src or anti-Fyn antibodies. A fraction of the precipitated kinases was allowed to phosphorylate acid-denatured enolase *in vitro* in the presence of [γ -³²P]ATP, followed by SDS-PAGE analysis and exposure to film (top panels). Another fraction of the immunoprecipitated material was analyzed by protein blotting for expression levels of Src or Fyn (bottom panels). Tyrosine phosphorylation levels of both kinases were unaltered (not shown). The experiment shown is representative of three performed.

Kv2.1 with cyt-PTP ϵ leads to a marked decrease in Kv current amplitudes, with 46 and 76% inhibition, respectively (Figure 5A–D). Similar to conclusions from our primary Schwann cell experiments, the inhibitory action of PTP ϵ does not involve significant changes in the kinetics or in the voltage dependence of channel activation (Figure 5A and B). PTP ϵ catalytic activity is important for this process, as a catalytically inactive, substrate-trapping mutant (Flint *et al.*, 1997) of cyt-PTP ϵ (D245A cyt-PTP ϵ) inhibits only 27% of Kv current amplitude, compared with 76% inhibition by wild-type cyt-PTP ϵ (Figure 5D and F). Residual inhibition by D245A cyt-PTP ϵ may result from binding of the inactive phosphatase to Kv2.1 (see below) in a manner which, though not dephosphorylating Kv2.1, may nonetheless impede its activity. These data are in line with those obtained with *Ptpre*^{-/-} mice and indicate that cyt-PTP ϵ can down-regulate activity of Kv channels by reducing tyrosine phosphorylation of their α -subunits. Consistent with these results, the tyrosine phosphatase inhibitor dephostatin causes up-regulation of K⁺ currents produced by co-expression Kv2.1 and cyt-PTP ϵ in *Xenopus* oocytes

(Figure 5E). In HEK 293 cells, cyt-PTP ϵ does not depress the basal Kv2.1 current activity (Figure 6A, B and F). This feature differs from *Xenopus* oocyte expression and is probably due to the lack of basal tyrosine phosphorylation of Kv2.1 channel α -subunits in HEK 293 cells, as revealed by our immunoprecipitation experiments (Figure 6H). However, when tyrosine phosphorylation of Kv2.1 α -subunits is stimulated by co-expressing activated Fyn kinase (Y531F), the Kv2.1 K⁺ current amplitude is up-regulated by >2-fold (617 ± 58 pA/pF, $n = 12$, $p < 0.01$) when compared with control Kv2.1-expressing cells (298 ± 63 pA/pF, $n = 15$) (Figure 6A, C, F and I). Under these stimulatory conditions, co-expression of cyt-PTP ϵ leads to a profound down-regulation of Kv2.1 current amplitude (101 ± 28 pA/pF, $n = 14$, $p < 0.001$). cyt-PTP ϵ totally suppresses the Fyn kinase stimulation of Kv2.1 current and tyrosine phosphorylation (Figure 6C, D, F and I). Similar results were obtained when Kv2.1 tyrosine phosphorylation was stimulated by activated Src kinase (Figure 6H). Correlative western blots show that co-expression of either activated Fyn kinase, activated Src kinase or cyt-PTP ϵ does not alter significantly the expression levels of Kv2.1 channel α -subunits (Figure 6G–I; see also Figure 7A).

Kv2.1 binds the active site of cyt-PTP ϵ

The above results suggest that cyt-PTP ϵ binds and dephosphorylates α -subunits of Kv channels. In agreement with this model, several-fold more Kv2.1 co-precipitate with a D245A substrate-trapping mutant (Flint *et al.*, 1997) of cyt-PTP ϵ than with wild-type cyt-PTP ϵ (Figure 7A). Significantly, enhanced binding to D245A cyt-PTP ϵ does not occur in the presence of sodium pervanadate (Figure 7A), which irreversibly oxidizes the conserved cysteine residue located at the active site of tyrosine phosphatases (Huyer *et al.*, 1997). Oxidation of this cysteine residue by pervanadate is known to disrupt active site-mediated binding of D-to-A type phosphatase mutants to their putative substrates (Flint *et al.*, 1997; Huyer *et al.*, 1997). These results indicate that Kv2.1 interacts mainly with the active site of cyt-PTP ϵ , consistent with Kv2.1 being a substrate of cyt-PTP ϵ . An alternative model based on findings in PTP α -deficient mice (Ponniah *et al.*, 1999; Su *et al.*, 1999) suggests that cyt-PTP ϵ could indirectly control phosphorylation of Kv channel α -subunits by regulating activities of Src family tyrosine kinases. Fyn is the major Src-family tyrosine kinase active towards Kv channels in Schwann cells (Sobko *et al.*, 1998a); yet, expression, activity and overall tyrosine phosphorylation of both Fyn and Src are unchanged in *Ptpre*^{-/-} Schwann cells (Figure 7B and data not shown). Although this result does not rule out interactions between Fyn/Src and cyt-PTP ϵ , it appears that these kinases are not the major mediators of cyt-PTP ϵ activity towards Kv channels in the system studied here.

Discussion

Results presented here indicate that lack of cyt-PTP ϵ expression results in reduced myelination of sciatic nerve axons in early post-natal *Ptpre*^{-/-} mice. This finding parallels increased activity of voltage-gated potassium channels in primary Schwann cells derived from these

mice, which in turn is mediated by hyperphosphorylation of Kv channel α -subunits. *Ptpre*^{-/-} Schwann cells modulate phosphorylation of Kv1.5 and Kv2.1 differently than wild-type cells in response to broad-spectrum inhibition of tyrosine phosphatases or kinases, further attesting to the importance of this PTPase in early post-natal Schwann cells. The ability of cyt-PTPε to reduce phosphorylation and to down-regulate Kv1.5 and Kv2.1 can be reproduced in heterologous expression systems. Taken together, these findings indicate that cyt-PTPε dephosphorylates α -subunits of delayed-rectifier Kv channels, and that lack of cyt-PTPε most likely causes Schwann cell dysfunction *in vivo* in young mice. This study provides the first *in vivo* evidence of a functional link between a specific tyrosine phosphatase and regulation of Kv channel activity, and clearly defines its physiological consequences.

Although the above data suggest that cyt-PTPε plays a significant role in regulation of Kv phosphorylation in Schwann cells, the fact that myelination of sciatic nerve axons is normal in adult *Ptpre*^{-/-} mice indicates that other PTPases participate in this process as well. The need for cyt-PTPε for proper Schwann cell function then likely decreases as mice age. A possible candidate for compensating lack of cyt-PTPε in Schwann cells is PTPσ, as young mice lacking PTPσ exhibit reduced myelination of sciatic nerve axons caused by a yet undetermined molecular mechanism (Wallace *et al.*, 1999; M.Tremblay, personal communication). Nonetheless, the existence of myelination defects in post-natal *Ptpre*^{-/-} mice demonstrates that cyt-PTPε performs a unique function in Schwann cells of such mice, and that other phosphatases cannot replace this enzyme at this developmental stage.

Recent data have shown that K⁺ channel activity can be either up-regulated or down-regulated by PTKs depending on the physiological context. Heterologous expression studies indicate that the K⁺ channel activity of several Kv1 family members is down-regulated by tyrosine kinases. In HEK 293 cells, phosphorylation of Kv1.3 by activated v-src or by epidermal growth factor treatment leads to a decrease in current amplitude (Holmes *et al.*, 1996; Bowlby *et al.*, 1997; Fadool *et al.*, 1997). The current amplitudes of several other Kv channels, such as Kv1.2 and Kv1.5, are known to be strongly suppressed by tyrosine kinases (Timpe and Fantl, 1994; Holmes *et al.*, 1997; Tsai *et al.*, 1997). However, a recent *in vitro* study demonstrates that tyrosine phosphorylation can up-regulate the activity of the Kv1.1 channel (Wang *et al.*, 1999). Differential regulation of K⁺ channel activity by tyrosine phosphorylation has also been observed *in vivo*. Activation of the insulin receptor inhibits Kv current amplitude in olfactory bulb neurons (Fadool and Levitan, 1998). On the other hand, positive modulation of transient K⁺ channels by PTKs was described in rat ventricular cardiac cells (Guo *et al.*, 1995). Ca²⁺-activated K⁺ channels are up-regulated by PTK activation in mouse fibroblasts and in Chinese hamster ovary cells (Prevarskaya *et al.*, 1995; Decker *et al.*, 1998). More recently, ceramide was found to increase the delayed-rectifier K⁺ current via PTK activation in cultured cortical neurons, and this process parallels increased apoptosis of these cells (Yu *et al.*, 1999). Similarly, our recent work showed that the Fyn

tyrosine kinase increases delayed-rectifier K⁺ channel activity in mouse Schwann cells, and that exposure to tyrosine kinase inhibitors markedly down-regulates Kv current amplitude and inhibits cell proliferation in this system (Sobko *et al.*, 1998a,b).

The intimate connections between activities of PTKs and PTPases argue that dephosphorylation of Kv channels may also affect channel activity in a manner dependent upon the circumstances of the reaction. Interestingly, *in vitro* expression of PTPα, a phosphatase closely related to the transmembrane, receptor-like form of PTPε, has been shown to correlate with activation of Kv1.2 (Tsai *et al.*, 1999), the opposite of the effect that cyt-PTPε has on Kv1.5 and Kv2.1. This result may reflect basic differences in the manner by which PTPε and PTPα function, or in the effect dephosphorylation has on different Kv α -subunits. Alternatively, as our results are based on both *in vivo* (Schwann cells) and heterologous *in vitro* (*Xenopus* oocyte, HEK 293 cells) studies, this discrepancy may reflect differences in the molecular backgrounds of native versus heterologous cells and their influence on PTPα activity.

Our findings indicate that Kv channel phosphorylation in Schwann cells is regulated by opposing activities of tyrosine kinases and tyrosine phosphatases. One could then expect that inhibition of tyrosine kinases by genistein would offset Kv channel activation normally observed in *Ptpre*^{-/-} Schwann cells. This effect is indeed observed upon treatment of *Ptpre*^{-/-} Schwann cells with genistein; however, the magnitude of the effect is very mild. This may reflect residual kinase activity, which is not inhibited by genistein and which is now detectable in the absence of PTPε. Alternatively, blockade of tyrosine kinases by genistein may be less effective in the context of defective regulation of tyrosine dephosphorylation, which exists in *Ptpre*^{-/-} Schwann cells.

The precise link between Kv channel activity and Schwann cell-mediated myelination is unclear at the present time. However, there is temporal correlation between decreases in activities of Src-related tyrosine kinases and of Kv channels, on the one hand, and exit of Schwann cells from the cell cycle and onset of myelination, on the other hand (Sobko *et al.*, 1998a,b; MacFarlane and Sontheimer, 2000). Our data indicate that lack of PTPε results in increased tyrosine phosphorylation and activities of Kv channels, possibly delaying exit of *Ptpre*^{-/-} Schwann cells from the cell cycle and resulting in delayed myelination.

In all, results presented here indicate that, together with the Src family tyrosine kinases, cyt-PTPε is part of the finely tuned molecular mechanism that regulates Kv channel activity during Schwann cell development and myelination of peripheral nerves *in vivo*. Schwann cell dysfunction and myelination defects are known to be associated with severe human neurological diseases. Better understanding of how Schwann cell functions are regulated at the molecular level could increase the chances of eventually controlling such diseases.

Materials and methods

Gene targeting and genotyping

Two recombinant phage-containing fragments of mouse genomic DNA from the *Ptpre* locus were isolated from a strain 129 mouse genomic

library (Stratagene) using a fragment of the mouse *tm-PTP* cDNA (Elson and Leder, 1995a). The intron-exon structure of selected regions of these clones was determined and was found to be identical to that of *PTP α* exons 12–17 (Wong et al., 1993), with the PTPase signature motif spanning exons analogous to *PTP α* exons 13 and 14 (not shown). The targeting construct (Figure 1A) was based on the pPNT vector (Tybulewicz et al., 1991), with the Neo^r expression cassette of pPNT replacing a 2.9 kb PTPe genomic sequence containing exons 13–15. Following linearization and electroporation into TC1 ES cells (Deng et al., 1996), genomic DNA from ES cell clones that survived G418 and FIAU selection was analyzed by Southern blotting and PCR (not shown). Cells from two ES cell clones heterozygous for the targeted mutation were injected into C57BL/6 blastocysts, leading to chimeric mice and subsequently to germline transmission of the mutant allele. Mice were genotyped using DNA from tail biopsies by the Southern blot technique as shown in Figure 1B. All mice used in this report were of a mixed 129X57BL/6 background.

Cell culture

Primary Schwann cells were prepared from 3- to 5-day-old WT (*Ptpre*^{+/+}) or *Ptpre*^{-/-} pups as described (Sobko et al., 1998a). The young age of the pups prevented their individual genotyping prior to cell preparation, hence age-matched WT and *Ptpre*^{-/-} pups were obtained from separate matings of WT or of *Ptpre*^{-/-} mice in which all pups were of the desired genotype. Sciatic nerves obtained from mice of a given litter were pooled prior to cell preparation. HEK 293 cells were grown and transfected by the calcium phosphate technique as described (Toledano-Katchalski et al., 1999). In some cases HEK 293 cells were transfected with vectors expressing rat Kv2.1 (gift of Drs J. Barhanin and M. Lazdunski), chicken Y527F Src and human Fyn Y531F (gifts of Dr S. Courtneidge), or mouse cyt-PTP (Toledano-Katchalski et al., 1999).

Electrophysiology

Macroscopic whole-cell currents were recorded in Schwann cells or in transfected HEK 293 cells, as previously described (Sobko et al., 1998a). HEK 293 cells were co-transfected with pIRES-CD8 (kindly provided by Drs J. Barhanin and A. Patel, CNRS, Sophia Antipolis, France) as a marker for transfection. Transfected cells were visualized 40 h following transfection, using the anti-CD8 (Dyna) antibody-coated bead method (Jurman et al., 1994). Signals were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments), sampled at 2 kHz and filtered below 0.8 kHz via a four-pole Bessel low pass filter. Data acquisition was carried out using pClamp 6.0.2 software (Axon Instruments). The patch pipettes with tip resistance of 4–8 M Ω were filled with (in mM): 164 KCl, 2 MgCl₂, 1 CaCl₂, 11 EGTA, 10 HEPES, 11 glucose at pH 7.4. The external solution contained (in mM): 140 NaCl, 5 KCl, 5 CaCl₂, 2 MgCl₂, 11 glucose and 10 HEPES at pH 7.4. Series resistances were within 8–16 M Ω and were compensated by 85–90%. The sustained component of the K⁺ currents was measured at the end of the depolarizing traces. All data were expressed as mean \pm SEM. Statistically significant differences were assessed by Student's *t*-test.

For heterologous expression in *Xenopus laevis*, stage V and VI oocytes were used to inject cRNA, as described previously (Abitbol et al., 1999). Capped complementary RNAs (cRNAs) were injected at 2.5 ng cRNA/oocyte. Standard two-electrode voltage-clamp measurements were performed 3–5 days following cRNA microinjection into oocytes as described (Abitbol et al., 1999). Current signals were filtered at 0.5 kHz and digitized at 2 kHz. Chord conductance (*G*) was calculated using the following equation: $G = I/(V - V_{rev})$ where *I* corresponds to the current amplitude and *V_{rev}* is the measured reversal potential, assumed to be -90 mV (-90 \pm 2 mV; *n* = 7). *G* was estimated at various test voltages *V* and then, normalized to a maximal conductance value *G_{max}*, calculated at +30 mV. Activation curves were fitted by a Boltzmann distribution: $G/G_{max} = 1/[1 + \exp\{(V_{50} - V)/s\}]$, where *V₅₀* is the voltage at which the current is half-activated and *s* is the slope factor.

Protein blot analysis

Selected organs and primary Schwann cells were homogenized in buffer A (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1% NP-40), supplemented with 0.5 mM sodium pervanadate and protease inhibitors (Sigma), and analyzed as described (Elson and Leder, 1995a). Antibodies used in this study included polyclonal anti-PTPe (Elson and Leder, 1995a), monoclonal (clone D4/11) or polyclonal anti-Kv2.1 (Upstate Biotechnology), polyclonal anti-Kv1.5 (Upstate Biotechnology and Alomone Labs), monoclonal anti-Fyn (Santa Cruz Biotechnology), monoclonal anti-v-Src (Calbiochem), anti-FLAG M2 affinity beads

(Sigma) or anti-phosphotyrosine (clone PY20; Transduction Laboratories).

Immunoprecipitation and substrate-trapping experiments

The D245A mutation was introduced into the mouse cyt-PTP cDNA (Elson and Leder, 1995b) by site-directed mutagenesis; the presence of the desired mutation and absence of other mutations were verified by DNA sequencing. PTPe cDNAs all contained a FLAG tag at their C-termini and were expressed from the pCDNA3 vector (Invitrogen). Wild-type cyt-PTPe, but not D245A cyt-PTPe, was catalytically active towards paranitrophenyl phosphate (not shown). For immunoprecipitations, cells were lysed in buffer A supplemented with 5 mM iodoacetic acid and protease inhibitors. Cellular proteins (0.5–1 mg) were reacted with the relevant antibodies for 6–8 h, followed by four extensive washes with RIPA buffer. When used, pervanadate (0.5 mM) replaced iodoacetate in the lysis buffer.

Electron microscopy

Three 5-day-old mice of either genotype were studied. Nerve fibers were fixed in 3% paraformaldehyde/2% glutaraldehyde and processed for electron microscope analysis as described (Shinder and Devor, 1994). Samples from different mice were always taken from the same location, 5 mm from the proximal end of the nerve. Vertical cross-sections 70–90 nm thick were cut with a Leica Ultracut UCT ultra-microtome, stained with uranyl acetate and lead citrate, and examined in a Philips 410 transmission electron microscope at 100Kv. Five to seven non-overlapping cross-section fields were examined from each mouse at 2800 \times magnification. The thicknesses of myelin sheaths of all axon profiles in a given field were measured, and data for all mice of the same genotype were pooled. Similar experiments were conducted on sciatic nerve tissue obtained from 8-month-old male mice (three mice from either genotype).

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